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Ruminant Immunity to Abomasal Parasites

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Declaration

I declare that this thesis has been composed by me, Aileen Halliday, and that I have made a substantial contribution to the work as indicated at the start of each chapter. This work has not been submitted in whole or in part for any other degree or professional qualification.

Aileen Murray Halliday

June 2013

Abstract

The studies submitted herein have contributed to our understanding of ruminant immunology, host-parasite interactions during ruminant infection with nematode parasites, and potential vaccine strategies to combat parasitic gastroenteritis (PGE). PGE of sheep and cattle, caused by *T. circumcincta* and *O. ostertagia* respectively, is a major problem for the global farming industry both in terms of productivity and animal welfare. To date control of these parasites has relied on the use of anthelmintic drugs however the emergence of widespread anthelmintic resistance is driving the search for alternative methods of control. As ruminants do acquire immunity in the field, vaccination is one such alternative under investigation.

The first three papers contributing to this thesis used modern immunological tools alongside a locally developed surgical technique to revisit a model of nematode infection in sheep, investigating the composition and kinetics of the ovine local immune response to infection with *Trichostrongylus circumcincta* via cannulation of the efferent gastric lymph duct. A protective local secondary immune response was observed in sheep which had previously experienced infection with *T. circumcincta*, but was absent from naive sheep. This immune response consisted initially of a rise in T- and B- cell activity peaking at 3 and 5 days post challenge respectively, followed by a secondary parasite-specific IgA response from 5 days post challenge which correlated with stunting of parasite growth. Significant parasite loss occurred by 2 days post challenge, prior to detection of the secondary immune response, suggesting critical early events in the host-parasite interaction and the potential importance of larval antigens in

these interactions. No difference was observed in either the manifestations of immunity, or the magnitude and quality of the immune response, between adult sheep and lambs.

The fourth and fifth papers describe vaccine trials carried out in bovine and ovine hosts using detergent soluble proteins derived from 4th larval stage *Ostertagia ostertagi* and *Teladorsagia circumcincta* respectively as antigens. Substantial reduction in total faecal egg output of up to 85% was observed in the calf trials, but not in the sheep trials which attained a maximum reduction of 29% in total faecal egg output.

The sixth paper is a transcriptomic study carried out using the Roche 454 sequencing platform to investigate the immediate responses of *Teladorsagia circumcincta* upon encountering ovine host tissue of either immune or naive status. Following larval exsheathing and 4 hours of exposure to either immune or naive abomasal environments the transcript level of several genes was observed to differ. Genes which were most upregulated in response to encountering the immune environment included a peptidyl-glycine alpha-amidating mono-oxygenase homologue and a small heat shock protein.

The studies described herein represent a body of work carried out using up-to-date tools and technologies. The first three papers confirmed the existence of critical early events in the host-parasite interaction, pointing to the potential use of larval antigens as vaccine candidates described in the trials in papers 4 and 5, and leading to the in-depth transcriptomic analysis described in paper 6. Papers 4 and 5 demonstrated that while *Teladorsagia circumcincta* and *Ostertagia ostertagi* have similar life cycles and host-site predilection, and both

the ovine and bovine host can develop immunity to incoming parasitic larvae in the field, important differences may exist in either the proteome of the fourth stage larvae and/or the nature of the host response. Paper 6 revealed that changes in *T. circumcincta* transcript levels in response to ovine-host immune status can be detected early in the host-parasite interaction.

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Abbreviations

AAD	Amino-acetonitrile derivative
Al(OH) ₃	Aluminium hydroxide
ANOVA	Analysis of variance
ASP	Ancylostoma secreted protein
Bh	Beryllium hydroxide
BLAST	Basic Local Alignment Search Tool
CD	Cluster of Differentiation
cDNA	complementary DNA
ConA	Concanavilin A
DNA	Deoxyribonucleic Acid
<i>E. coli</i>	<i>Escherichia coli</i>
EBSS	Earles buffered saline solution
ELISA	Enzyme linked immunosorbent assay
ES	Excretory-secretory
EST	Expressed sequence tag
EU	European Union
FACS	Fluorescent activated cell sorting
FCA	Freund's complete adjuvant
FEC	Fecal egg counts
FIA	Freund's incomplete adjuvant
GL	Globule leukocytes
H-Gal-GP	<i>Haemonchus</i> galactose-containing glycoprotein complex
<i>H. contortus</i>	<i>Haemonchus contortus</i>
HSP	Heat shock protein
I.M.	Intramuscular
I.P.	Intraperitoneal
I.V.	Intravenous
IFN- γ	Inteferon gamma

Ig	Immunoglobulin
IgA	Immunglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
KEGG	Kyoto Encyclopedia of Genes and Genomes
L1	First larval stage
L2	Second larval stage
L3	Third larval stage
L4	Fourth larval stage
L5	Fifth larval stage
LC-ESI-MS/MS mass spectrometry	liquid chromatography-electrospray ionization-tandem mass spectrometry
LMI	Larval migratory inhibitory
MMC	Mucosal mast cells
MOWSE	Molecular Weight Search
NCBI	Naitonal Center for Biotechnology Information
<i>O. Ostertagi</i>	<i>Ostertagia ostertagi</i>
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Parasitic gastroenteritis
PPR	Periparturient Rise
Quil A	Quil A purified Saponin
RNA	Ribonucleic Acid
S.C.	Subcutaneous
SCP	Sperm coating protein
SDS-PAGE electrophoresis	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMCP	Sheep mast cell protease
<i>T. circumcincta</i>	<i>Teladorsagia circumcincta</i>
<i>T. colubriformis</i>	<i>Trichostrongylus colubriformis</i>
TGF beta	Transforming growth factor beta

Th2	T-helper type 2
TNF	Tumour necrosis factor
TSBP	Thiol sepharose binding proteins

1 Introduction

1.1 Gastrointestinal parasitic nematodes

Parasitic nematodes are highly prevalent in ruminants throughout the world, with over 70 species identified of which over thirty were isolated from the gastrointestinal tract of small ruminants (Taylor *et al* 2007). The most pathologically important species are of the family *Trichostrongylidae*, with the most common genera in the United Kingdom being *Ostertagia ostertagi* in cattle and *Haemonchus contortus*, *Teladorsagia circumcincta* and *Trichostrongylus* species in sheep (Boag and Thomas 1975, Burgess *et al* 2012; Gibbs 1982).

1.2 *Teladorsagia circumcincta* and *Ostertagia ostertagi*

1.2.1 Life Cycle

Teladorsagia circumcincta and *Ostertagia ostertagi* are parasitic nematodes of the order *Strongylida*. They have a direct life-cycle, with a host-site predilection for the abomasa of sheep and cattle respectively (Figure 1.1).

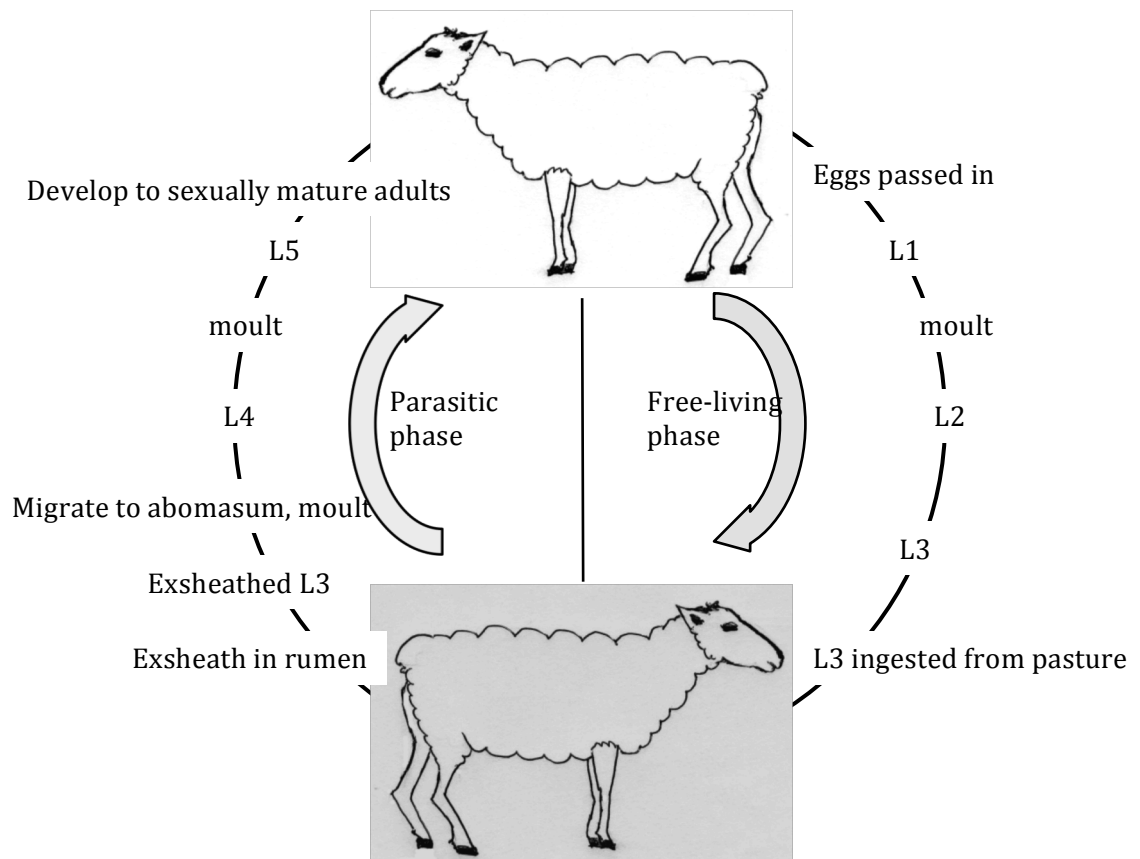


Figure 1.1 Life cycle of *Teladorsagia circumcincta*.

Eggs are passed in the faeces onto pasture, and development through the L1 and L2 stages to infective 3rd stage larvae (L3) occurs in the faecal pat. Infective 3rd stage larvae migrate onto herbage in moist conditions. The infective L3 larvae are the most resistant to dessication of the free living stages, retaining the cuticle from the L2 stage as a protective sheath. Infective L3s are ingested with herbage, after which they exsheath in the rumen then migrate to the abomasum where they invade the mucosa. The larvae penetrate the gastric glands and further development occurs within the gastric pit causing extensive

damage, before the larvae emerge from 7 - 21 days later as 5th stage larvae.

Infective larvae which are ingested at a time when environmental conditions would not be favourable for the hatching and development of their offspring, such as during autumn and winter in northern climates, can enter a state of hypobiosis (temporary arrested development) (Anderson *et al* 1965; Eysker 1981; Langrova *et al* 2008; Reid and Armour 1972) and this can persist for periods of up to 6 months (Sommerville 1953). This inhibited development has been linked to infection rate, with a higher proportion of worms becoming arrested in larger infections (Dunsmore 1960). As noted above, this can be attributed to seasonal factors with larvae ingested in late autumn/winter being more likely to become arrested. Worm populations in ewes have been shown to consist of mainly immature L4 in winter but adults in the spring (Connan 1968; Reid and Armour 1972). Resumption of development occurs when environmental conditions are more favourable for larval survival on pasture, and may be cued by external environmental changes or may be under parasite genetic control (Frank *et al* 1988). The Periparturient Rise (PPR) discussed below is often attributed to the maturation and fecundity of these arrested larvae.

Subsequent maturation into adult worms and mating takes place on the surface of the abomasum, with eggs secreted in the faeces from 16-18 days following ingestion. The entire life cycle can take place in 4-6 weeks (Armour *et al* 1966; Denham 1969; Myers and Taylor 1989; Rose 1969; Taylor *et al* 2007).

1.2.2 Epidemiology

There are 4 main factors which precipitate loss of production associated with helminth infection (Armour 1980). These are an increase in pasture larval contamination, altered susceptibility of existing stock, susceptible stock being introduced into a contaminated area and introduction of infection to a previously clean environment. Climatic condition, in particular wet weather, is the main factor increasing the infective mass on pasture (Waller and Thomas 1978). Larvae which have over-wintered, either on pasture or as hypobiotic larvae in the abomasa of infected animals, infect young animals in early spring-time. During the peri-parturient relaxation in immunity susceptibility of the female host is altered which leads to an increase in the number of eggs being laid down onto pasture by these animals (Barger 1993; Hammerberg and Lamm 1980; Wilson *et al* 2008). First season grazing animals continue to ingest infective larvae which in turn develop into egg-laying adults and larval numbers on pasture build up over summer to peak in the autumn before declining again. In temperate areas, there is a peak in faecal *T.circumcincta* egg output by ewes between April and June. This is known as the periparturient rise (PPR) and occurs 6-7 weeks after the start of lambing, just prior to weaning and lasts for 6-8 weeks (Brunsdon 1964; Crofton 1958). As a result, high numbers of infective larvae are on the pasture during mid-summer when susceptible young lambs begin grazing (Gibson and Everett 1973). The PPR has been attributed to a

number of factors including nutritional status of the dam (Houdijk *et al* 2000) and the onset of lactation (Jeffcoate *et al* 1992).

Type I teladorsagiasis / ostertagiasis occurs in young sheep or cattle, and is caused by the presence of larvae growing and developing in the abomasal glands, generally occurring during the first grazing season. Type II disease, which is more common in cattle than in sheep, occurs in yearlings or older animals and is caused by the simultaneous resumption of development of large numbers of hypobiotic larvae residing in the gastric glands (Myers and Taylor 1989; Reid and Armour 1973).

Lambs develop immunity to *T. circumcincta* within the first year, whereas immunity to *O. ostertagi* in cattle is slower to develop and can take up to 2 years (Klesius 1988; Michel 1963; Seaton *et al* 1989; Waller and Thomas 1978).

1.2.3 Pathogenesis

Sub-clinical infection with *T. circumcincta* or *O. ostertagi* can cause production losses, while the principal clinical effects of infection are diarrhoea, inappetance, elevated blood pepsinogen and gastrin levels, impaired abomasal function, alterations in protein metabolism, reduced growth, changes in body composition, emaciation and high morbidity, with implications for both animal welfare and productivity (Armour *et al* 1966; Fox 1993). The estimated cost to the British farming industry through gastrointestinal parasites alone is £84 million annually, with *Teladorsagia circumcincta* and *Ostertagia ostertagi* contributing to a large proportion of that (Nieuhof and Bishop 2005).

Incoming larvae of both *T. circumcincta* and *O. ostertagi* invade the gastric glands in the abomasum of sheep and cows respectively, after which they undergo a period of rapid growth. This creates nodular lesions in the tissue, caused both by mechanical stretching of the glands due to rapid larval growth and also by host inflammatory responses, resulting in mucosal cell hyperplasia and distension of the gland, before the parasites re-emerge on to the surface of the abomasum. During this period of growth damage is caused to the acid-producing parietal cells resulting in replacement with non-differentiated non-acid producing cells, an increase in abomasal pH and impaired abomasal function (Scott *et al* 1998a; Scott *et al* 2000). The pH of the abomasal fluid can rise from an uninfected range of 2-3.5 to a maximum of up to 7.5 (McLeay *et al* 1973; Scott *et al* 2000). The activation of the digestive enzyme, pepsinogen, to pepsin requires a pH of less than 4 and the activity of pepsin is optimal at around pH 2 (Schlamowitz and Peterson 1959). Thus, digestion is impaired. Physical damage is also caused to the abomasal tissue through destruction of cellular junctions, resulting in loss of plasma proteins such as albumin through the gastrointestinal tract leading to hypoalbumineamia (Fox *et al* 1989; Parkins *et al* 1990). Increased blood gastrin and pepsinogen levels are observed during parasite infection, with hypergastrineamia possibly stimulating increased proliferation of gastrin pro-genitor cells and a thickening of the mucosa (Fox 1993; Jain and Samuelson 2006; McLeay *et al* 1973; Parkins *et al* 1990; Scott *et al* 1998b; Scott *et al* 1999).

There is also evidence that the pathophysiology, and production losses, arising from infection with *T. circumcincta* in lambs can be largely attributed to the host

immune response. Greer et al (Greer *et al* 2008) demonstrated that immuno-suppression of young sheep during infection with *T. circumcincta* resulted in reduced mast cells, globule leukocytes and IgA in sequential abomasal tissue biopsies, as well as greater faecal egg counts. However, immuno-suppressed animals also had greater food intake and efficiency of energy utilisation, that is, greater weight gain, than infected non immune-suppressed animals.

1.2.4 Current Control Strategies

Current control strategies for both *T. circumcincta* and *O. ostertagi* rely on anthelmintic drug use combined with pasture management. Five classes of broad-spectrum anthelmintic are available: benzimidazoles/pro-benzimidazoles (eg. Fenbendazole); imidazothiazoles/tetrahydropyrimidines (eg. Levamisole); spiroindoles (eg. Derquantel), macrocyclic lactones (avermectins and milbemycins eg Ivermectin and Moxidectin) and amino-acetonitrile derivatives (AADs; Monepantel). The benzimidazoles have been the most commonly used class of anthelmintics. Benzimidazoles inhibit microtubule development, these being involved in a variety of vital cellular processes such as the secretory vesicle transport system. Levamisole is a nicotinic agonist which affects the parasite's nervous system. Unnatural excitation is caused when the drug binds to nicotinic acetylcholine receptors on the surface of the somatic muscles (Martin *et al* 1997). Derquantel is a nicotinic cholinergic antagonist and acting on somatic muscle nicotinic acetylcholine receptors to cause flaccid paralysis (Epe and Kaminsky 2013, Woods *et al* 2012). The macrocyclic lactones

are allosteric modulators of glutamate-gated chloride channels, affecting the somatic muscles of the worm causing paralysis which leads to worm expulsion, and also having effects on feeding and sensory inputs into behavior (Kohler 2001, Robertson *et al* 2012). The AADs interact with nicotinic acetylcholine receptors associated with neurotransmission (Kaminsky *et al* 2008a; Prichard and Geary 2008). Anthelmintic resistance was first reported for benzimidazoles, imidazothiazoles and macrocyclic lactones within approximately 10 years of release, and has been reported in both *T. circumcincta* and *O. ostertagi* including isolates resistant to multiple classes (Bartley *et al* 2004; Gasbarre *et al.* 2004; Sargison *et al* 2005; Sutherland and Leathwick 2011; Waller 1994). Amino-acetonitrile derivatives are relatively new to the market and strategies are being tested to slow the development of resistance to this class of anthelmintic (Dobson *et al* 2011; Kaminsky *et al* 2008b).

The wide-spread emergence and increasing prevalence of anthelmintic resistance is driving the search for complementary and alternative methods of control (Papadopoulos *et al* 2012). Vaccination, breeding of helminth-resistant sheep, manipulation of protein nutrition and non-conventional anthelmintics such as nematophagous fungi, copper-oxide wire particles and bio-active forages such as chicory are all alternative strategies under investigation for the control of *T. circumcincta* (; Bang *et al* 1990; Bisset *et al* 1996; Houdijk *et al* 2005; Knox 2000; Tzamaloukas *et al* 2006), reviewed by Hoste and Torres-Acosta (2011). Genetic factors, host-immunity and non-conventional control strategies such as bioactive forages are also being considered for the control of

O. ostertagi in cattle (Gasbarre *et al* 1990; Novobilsky *et al* 2011; Rinaldi and Geldhof 2011).

Targeted selective treatment is another complementary strategy under investigation for control of both *T. circumcincta* and *O. ostertagi*, whereby only the animals which would benefit most from anthelmintic treatment are treated (Hoglund *et al* 2009; Kenyon *et al* 2009). Parasite dispersion within a flock or herd is highly skewed, with approximately 10% of the animals housing the majority of the parasites (Stear *et al* 1995a). An approach whereby these heavily infected animals are identified using a decision making model, which takes into account pasture mass and individual live-weight gain, is providing encouraging results with regard to maintaining flock/herd productivity whilst reducing traditional anthelmintic usage (Greer *et al* 2008).

1.3 Immunity to infection

1.3.1 Manifestations of immunity

The development of resistance to infection is controlled by many factors, including dam, sire, sex, date of birth, history of exposure to infection, concurrent infection and nutritional status (Coop and Kyriazakis 1999; Stear *et al* 1996). Manifestations of resistance to infection can be observed via 3 main changes: stunting of developing worms, a reduction in total worm burden, and

inhibited development of larvae at the early L4 stage (Michel 1963; Seaton *et al* 1989).

1.3.2 Retardation of worm growth

The first observed manifestation of immunity is retardation of incoming parasites. Growth-stunting and an associated reduction in fecundity of *T. circumcincta* starts after 4 weeks of continuous exposure to incoming larvae, and has been associated with a local IgA response to fourth stage larvae (Seaton *et al* 1989; Stear *et al* 1995b). Growth retardation and reduced fecundity of *O. ostertagi* is also observed, and is also associated with the local IgA response (Claerebout *et al* 1999b). Morphological changes are also observed in both *T. circumcincta* and *O. ostertagi* recovered from immune animals, with female worms displaying smaller vulval flaps (McKellar 1993; Michel *et al* 1972).

1.3.3 Reduction in worm burdens

A reduction in worm burdens is observed in immune animals and may be attained through exclusion and/or expulsion of incoming and developing parasites. This may be the result of changes in the composition and rheological properties of the mucus following previous exposure to the parasite (Jackson *et al* 2004; Rinaldi *et al* 2011). An IgE-mediated immediate hypersensitivity response has been observed in the abomasa of immune sheep experiencing a

challenge infection, with eosinophilia and mucosal mast cell accumulation, and also an increase in sheep mast cell protease (Huntley *et al* 1995; Miller 1996; Stevenson *et al* 1994). Adult *T. circumcincta* burden has been negatively correlated with abomasal globule leukocytes derived from mucosal mast cells (Bisset *et al* 1996; Stear *et al* 1995b). In cattle, *O. ostertagi* infection has also been associated with increased numbers of eosinophils, mast cells and globule leukocytes in the mucosa (Baker *et al* 1993; Claerebout *et al* 1996; Claerebout *et al* 1998b; Snider III *et al* 1988). There is evidence of a significant association between globule leukocytes and protective immune responses against *O. ostertagi* (Claerebout *et al* 1998b), however in other studies no such correlation was observed (Claerebout *et al* 1996; Claerebout *et al* 1998a).

1.3.4 Inhibited larval development

Immune animals also show a larger proportion of larvae inhibited at the early L4 stage of development. While inhibited development of larvae following invasion of the gastric pit may be the result of hypobiosis caused by seasonal or environmental factors, it can also be immunologically mediated by the host (Michel *et al* 1979; Smith 2007; Snider III *et al* 1981).

1.3.5 Acquired immunity

The following summarises knowledge of the main effectors of immunity against *T. circumcincta* in sheep.

Following an immediate hypersensitivity response immune sheep and cattle display an anamnestic response which includes expansion of parasite specific CD4+ T cells in the abomasal lymph node and a subsequent increase in parasite specific antibody secreting B cells (Almeria *et al* 1998; Balic *et al* 2003; Gasbarre 1994). Cytokine profiling of naïve and immune animals reveals that immune animals show a bias towards producing Th2 cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13 and TGF-beta in the abomasal lymph nodes (Claerebout *et al* 2005b; Craig *et al* 2007; Gasbarre *et al* 2001; Li *et al* 2006), however it is not a simple case of Th2 responses correlating with protection. Pro-inflammatory cytokines such as IFN- γ and TNF are also observed to increase, and the Th2 cytokine profile is not always observed in the abomasal mucosa. Cytokine profiling through analysis of transcript levels of cytokines in the abomasal lymph nodes of the immune sheep described in papers 1-3 herein revealed a trend towards a Th2 cytokine profile following challenge infection (Craig *et al* 2007).

A series of experiments carried out in the 1980s involving cannulation of the ovine efferent gastric lymph duct allowed observation of the kinetics of the local immune response and demonstrated the rapid expansion of lymphocytes and subsequent rise in local IgA production in previously infected sheep (Smith *et al* 1983a; Smith *et al* 1983b; Smith *et al* 1984; Smith *et al* 1987). It was also observed that younger lambs mounted a less effective cellular and humoral immune response than older sheep, which may contribute to younger animals being more susceptible to infection (Smith *et al* 1985). Adoptive transfer experiments utilising the same cannulation technique, in which lymphocytes

derived from the gastric lymph of immune sheep were transferred to naïve identical twins, demonstrated the protective capacity of the lymphocytes contained therein, while the continued immunity of sheep from which lymphocytes had been removed also demonstrated the importance of factors other than lymphocytes in maintaining immunity to *T. circumcincta* (Smith *et al* 1986).

Mast cells: Increased local concentrations of mucosal mast cells (MMC) and globule leukocytes (GL) are a common feature of gastrointestinal nematode infections particularly when the host has been subjected to previous infections with that parasite (Balic *et al* 2000b). A temporary increase in the pepsinogen content of the gastric lymph was concomitant to the release of sheep mast cell protease (SMCP) after challenge of previously infected sheep with *T. circumcincta* larvae (Huntley *et al* 1987; Smith *et al* 1984). This, and a negative correlation between worm counts and SMCP levels (Stevenson *et al* 1994), suggests a role for this enzyme in resistance to this nematode infection in sheep. Goats are less able to expel *T. circumcincta* infections than sheep and a comparison of *T. circumcincta* larval development in young goats and sheep with the same trickle infection regime showed that kids had higher numbers of globule leukocytes, lower mast cell numbers and higher worm burdens, but with a higher proportion of worms only reaching the early L4 stage of development, suggesting that mast cells and globule leukocytes may be involved in regulating worm burdens and development (Macaldowie *et al* 2003).

Mucus Production and Content: Mucus can impair worm movement due to its sticky, viscous texture, and the layer of mucus that coats the gastrointestinal tissue has been shown to exhibit a chemical larval migratory inhibitory (LMI) activity, particularly in previously immunised animals (Claerebout *et al* 1999a; Douch *et al* 1983). Ruminant mucus contains many factors that could be responsible such as antibodies, leukotrienes, histamine, prostaglandins and amines.

Eosinophilia: Eosinophils are short-lived cells with both phagocytic and secretory properties, generating substances such as leukotrienes and reactive oxygen species, and are associated with inflammatory reactions, reviewed by Rothwell (Rothwell 1989). Eosinophils have been shown to collect around the L3 of *H. contortus* (Rainbird *et al* 1998) and have been demonstrated adhering to *H. contortus* L3 *in vitro*, causing deterioration of the parasite's surface, immobilisation and eventually death. In goats, peripheral eosinophil numbers following challenge were significantly greater in responder than non-responder goats challenged with *T. circumcincta* with both abomasal and jejunal tissue eosinophil numbers significantly higher in responders (Patterson *et al* 1996). Eosinophil counts have been correlated with significant reductions in egg output when sheep over 3 months old are naturally infected with *T. circumcincta* (Stear *et al* 2002).

Lymphocytes: There is a great deal of conflicting evidence regarding the role of lymphocytes in the development of immunity to gastrointestinal nematodes due

to apparent differences between the responses to larvae and adults and different nematode species (Balic *et al* 2000a). As noted earlier, a large increase in lymphocyte output in the gastric lymph has been demonstrated in response to a challenge infection of 50,000 *T. circumcincta* L3 administered to previously infected sheep (Smith *et al* 1983a). This occurred during the first 5 days post-infection and was followed by a peak in IgA-containing cells one day later. It has also been established that transfer of lymphocytes between genetically identical sheep can confer a limited degree of immunity (Smith *et al* 1986).

Specific Antibodies

IgA: IgA has been linked to resistance to *T. circumcincta* in sheep and has been associated with reduced worm length, and therefore fecundity (Stear *et al* 1999). A negative correlation has also been recorded between worm length and IgA levels in the gastric lymph (Smith *et al* 1987). Smith *et al* (Smith *et al* 1985) also showed that, after repeat infection, IgA levels in the gastric lymph of young lambs were much lower when compared to older animals. In addition, anti-*T. circumcincta* IgA activity has been considered as a suitable marker of host resistance (Strain *et al* 2002).

IgG: In rodent models, a role for IgG has been implicated in rapid expulsion. For example, Arasu *et al* (Arasu *et al* 1994) showed that treatment with anti-*Trichinella spiralis* IgG conferred immediate protection against infection with this species. In rodents, IgG1 production is stimulated in the Th2 type response. In *Trichuris muris* infections in mice, blocking of IL-4 function results in a large increase in IgG2 levels leading to a chronic infection whereas IFN- γ depletion

raises IgG1 secretion resulting in expulsion (Else *et al* 1994). A rise in serum IgG1 and, to a lesser extent, IgG2 is generally observed in secondary infection of calves with *O.ostertagi* and sheep with *H.contortus* (Canals and Gasbarre 1990; Schallig *et al* 1995).

IgE: For some time, IgE has been strongly associated with immunity to helminth infections, particularly in schistosomiasis in humans reviewed by Capron & Dessaint (1992). IgE production is an essential part of the Th2-type response, being up-regulated by IL-4 and down-regulated by IFN- γ (Miller 1996). There is some argument over how beneficial IgE responses, and associated immediate hypersensitivity reactions, can be to the host. IgE is thought to be responsible for increased mucosal permeability, important in the pathogenesis of this type of disease, via activation of mast cells and basophils (Miller 1996).

Elevated total and ES-specific IgE levels have been reported in the serum of sheep 2-4 weeks after infection with *H.contortus* (Kooyman, Van Kooten *et al*, 1997). A negative correlation between worm burden and serum total IgE levels was also recorded, indicating involvement of IgE in protective immunity against *H.contortus* (Kooyman *et al* 1997). However, a similar study with *T.circumcincta* did not produce the same results with only low specific IgE levels being detected in a small number of the infected animals (Huntley *et al* 1998).

1.4 Host-parasite interactions

1.4.1 Host response to infection

The intimate physical nature of parasitic infection of sheep and cattle with *T. circumcincta* and *O. ostertagi* respectively indicates an intimate immunological relationship with the host. In addition to the immediate hypersensitivity, Th2 cytokines, cellular and humoral responses mentioned previously, ovine hosts alter the expression of proteins including intelectin (French *et al* 2009; French *et al* 2008), gelsolin-b (Goldfinch *et al* 2008), and ovine chitinase-3 like 1 (Knight *et al* 2007). Sheep intelectin-2 immunolocalises to abomasal mucus neck cells and gastric mucus surrounding developing *T. circumcincta* larvae (French *et al* 2008). Both ovine and bovine hosts change the biosynthesis and glycosylation pattern of mucins in response to infection with abomasal nematodes (Hoang *et al* 2010; Rinaldi *et al* 2011), and intelectin has been shown to co-purify with the ovine mucin Muc5a thereby potentially altering the rheological properties of the mucus and creating a physical barrier to parasite invasion (Pemberton *et al* 2011). Gelsolin can act as an actin scavenger and may bind free actin released from damaged cells to prevent actin-polymerisation and subsequent effects on mucus viscosity (Sun *et al* 1999), and may also have a role in regulation of mucus secretion from goblet cells (Ehre *et al* 2005; Vandekerckhove *et al* 1990). Chitinase like proteins are part of a group of molecules upregulated Th2 type immune responses, mediating effector functions and contributing to tissue remodelling (Lee *et al* 2011). A microarray study by Li *et al* (2010) identified 3 pathways significantly impacted during bovine infection with *O. ostertagi*: the complement system, leukocyte

extravasation and acute phase responses. Proteomic analysis of the efferent gastric lymph which was collected from either immune or naïve sheep during the experiments described in papers 1 and 2 of this thesis revealed significant changes in gelsolin (reduced in naïve sheep after primary infection), α -1 β glycoprotein (reduced after challenge of immune sheep) and the acute phase protein haemopexin (increased after challenge of immune sheep), which may reflect part of the local response to infection with *T. circumcincta* (Goldfinch *et al* 2008). An *in vitro* model designed to investigate the immune exclusion of *Teladorsagia circumcincta* from abomasal tissue detected the presence of galectin -14 and -15, and sheep mast cell protease 1 in mucosal washes prior to larval challenge, and after challenge, intelectin and calcium activated chloride channel were also present in the mucosal washings (Athanasiadou *et al* 2008). Galectin-15 protein is secreted into the lumen of sheep infected with *H. contortus* and is detected in the mucus where it may interact with carbohydrate motifs such as mucins (Dunphy *et al* 2000).

1.4.2 Parasite-mediated immunomodulation

There is also growing evidence of the immunomodulatory capabilities of *T. circumcincta* and *O. ostertagi* upon their hosts, which may be an adaptive response to evade the immune system and survive in the host by manipulating the environment in the host in favour of the parasite (Hewitson *et al* 2009). Secretory products of *T. circumcincta* induce Foxp3 expression in regulatory T

cells thereby possibly contributing to modulation of the host response (Grainger *et al* 2010), and *O. ostertagia* L4 secretory products inhibit bovine T lymphocyte responses (Gomez-Munoz *et al* 2004). Neutrophil and eosinophil chemotactic factors, a calcium activated apyrase and a macrophage migration inhibitory-like tautomerase have also recently been identified as potentially immunomodulatory proteins secreted by *T. circumcincta* and/or *O. ostertagi* (Nisbet *et al* 2010a; Nisbet *et al* 2011; Reinhardt *et al* 2011; Zarlenga *et al* 2010). The secretome of *T. circumcincta* and *O. ostertagi* larvae also includes activation-associated secreted proteins, the novel protein family containing the SCP/Tpx-1/Ag5/Pr-1/Sc7 motif unique to *T. circumcincta* and *O. ostertagi*, and metalloproteases, which may also play a role in larval establishment and evasion or modulation of the host immune response (Geldhof *et al* 2003; Nisbet *et al* 2010b; Saverwyns *et al* 2008; Smith *et al* 2009; Vercauteren *et al* 2003).

1.4.3 Parasite transcriptomics

Transcriptomic studies have been carried out on several parasitic nematode species, with many annotated EST datasets publicly available via, for example, NEMBASE4 (Elsworth *et al* 2011). The arrival of next-generation sequencing technologies such as the Roche 454-titanium platform (Roche 2011) and advances in bioinformatics have allowed increasingly extensive transcriptomic analyses. The transcriptomes of the ruminant nematode parasites *Haemonchus contortus*, *Dictyocaulus viviparus*, and *Trichostrongylus colubriformis* have all

been studied using these methods, both as a means of deep-sequencing the entire transcriptome and as a means of comparing parasites at different life stages (Cantacessi *et al* 2005; Cantacessi *et al* 2010a; Cantacessi *et al* 2010b). The technique has also been employed recently to investigate the responses of anthelmintic resistant *T. circumcincta* to anthelmintic treatment (Dicker *et al* 2011).

An investigation into the response of the nematode parasite *Strongyloides ratti* to host immune status was carried out by O'Meara *et al* (O'Meara *et al* 2010) using a microarray approach. This study revealed that the parasite transcriptome was relatively stable, with only a small subset of genes differing in transcription according to whether the parasite was encountering an immune or naïve host environment. Genes which were upregulated when the parasite encountered an immune host or high immune pressure included *ida-1* (a molecular component of the secretory machinery), a small heat-shock protein family member, *unc-54* (myosin heavy chain) and a c-type lectin family member. The results suggest these proteins may be involved in maintaining the parasite in a hostile environment, or may be involved in modulating the host immune response. Paper 6 in this thesis uses 454 sequencing technology to address a similar question during *Teladorsagia circumcincta* infection of sheep: What changes occur in the parasite transcriptome upon encountering either an immune or naïve ovine host environment?

1.5 Cannulation of the gastric lymph duct

As mentioned in section 1.3.5. (acquired immunity) a series of experiments carried out in the 1980s cannulating the efferent gastric lymph duct of sheep provided the first details of the kinetics of the ovine local immune response after primary and challenge infection with *Teladorsagia circumcincta* (Smith et al 1983a; Smith et al 1983b; Smith et al 1984; Smith et al 1987). A simplified representation of the location of the cannula and the components of the gastric lymph is shown in Figure 1.2.

These studies revealed that the lymphocytes of immune sheep respond faster than those of their naïve counterparts, determined by total output of large basophilic cells following parasite infection. A peak in large basophilic cells was observed at day 4 in immune sheep, but the frequency was not significantly higher in naïve sheep until days 7-9 (Smith *et al* 1983a; Smith *et al* 1983b). Total IgA levels were also observed to rise sooner in immune sheep and were significantly increased by 6 days post challenge, whereas in naïve sheep undergoing primary infection total IgA levels had not risen at the time of slaughter 3 weeks after infection. These studies provided a valuable insight into the manifestations of immunity and the kinetics of the immune response, however the technology to determine specific lymphocyte sub-populations and did not exist at that time.

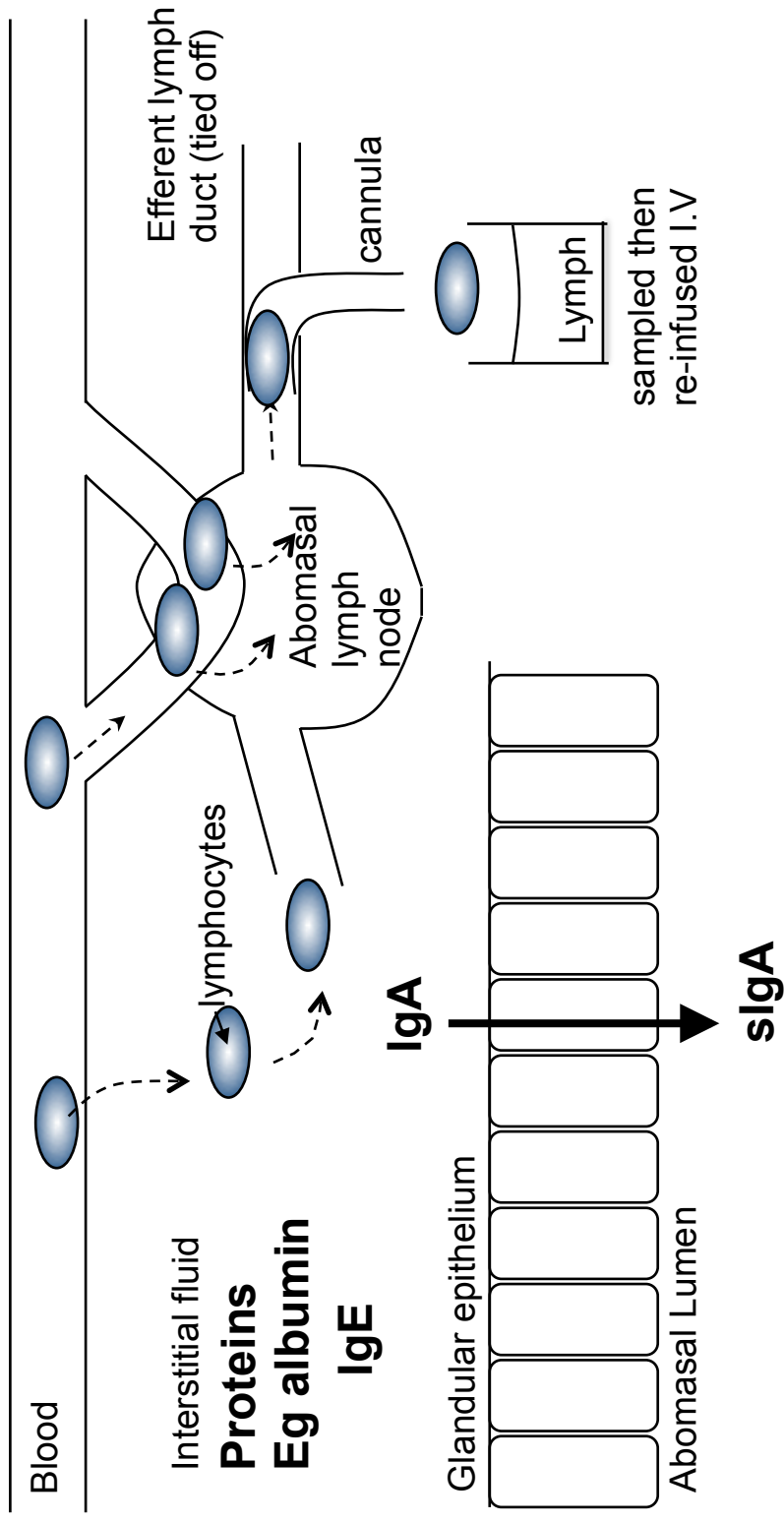


Figure 1.2 Simplified lymph physiology and collection of efferent gastric lymph.

More recently, studies have been carried out involving cannulation of the intestinal lymph duct of sheep and collection of efferent and pseudo-afferent lymph to study ovine responses to the intestinal nematode parasite *Trichostrongylus colubriformis*. The results revealed that genetically resistant sheep and immune sheep had higher total IgE in the afferent and efferent lymph, with genetically resistant sheep showing higher expression of IL-5, IL-13 and TNF-alpha in lymph cells and a strong polarisation to a Th2-type cytokine response (Pernthaner *et al* 2005a; Pernthaner *et al* 2005b). After infection, but prior to development of immunity to *T. colubriformis*, parasite-mediated down-regulation of many genes involved in immune function in lymph cells was observed, including genes involved in antigen presentation, endocytosis and protein ubiquitination – linked to intracellular signalling of immune cells in response to environmental stimuli (Knight *et al* 2010, Malynn and Ma 2010).

Papers 1, 2 and 3 in this thesis revisited the method developed at Moredun of cannulating the efferent gastric lymph duct and used modern immunological and molecular techniques to gain a more in-depth understanding of the ovine immune response and the development of immunity to *Teladorsagia circumcincta*.

1.6 Vaccine trials

There are very few vaccines commercially available against parasites of sheep and cattle, however successful vaccines do exist and vaccination against

gastrointestinal nematodes is an attractive prospect. Stimulating the animal's own immune system to combat parasitic infection would cut down substantially on anthelmintic usage, contributing to the goal of sustainable parasite control, slowing the spread of resistance to anthelmintic drugs and reducing concerns over toxic drug residues in the food chain. As sheep and cattle do acquire immunity to *T. circumcincta* and *O. ostertagi* respectively, albeit at different rates, vaccination is viewed as a promising potential alternative control strategy for these parasites (Claerebout *et al* 2003; Smith and Zarlenga 2006). Barnes *et al* (Barnes *et al* 1995) have suggested, through development of models, that a successful vaccine against nematode parasites would be required to confer between 60% - 80% protection on 80% of the stock, and worm control in weaned animals is of primary importance as these are the flock/herd members most affected by gastrointestinal parasites. Several vaccination strategies have been tested to date with variable success. Approaches have included vaccination with larvae which have been attenuated through irradiation, vaccination with conventional parasite antigens such as surface proteins or excretory/secretory (ES) products, and vaccination with hidden parasite antigens such as gut antigens.

1.6.1 Attenuated larvae

A successful live attenuated larval vaccine against the cattle lungworm *Dictyocaulus viviparus* has been available since the 1950s (Jarrett *et al* 1957a; Jarrett *et al*

1957b), currently marketed as Bovilis® Huskvac (MSD Animal Health 2006), and a similar approach is also used in India to vaccinate sheep and goats against the lungworm *Dictyocaulus filarial*, which is the most prevalent and pathogenic nematode in that region, responsible for heavy sheep mortality and morbidity (Sharma *et al* 1988, Sharma 1994). Vaccination of 2 month-old lambs or adult sheep with irradiated *H. contortus* resulted in significant protection against homologous challenge following 2 doses for the adults and 4 doses for the lambs (Smith and Angus 1980). A similar trend was observed following vaccination with irradiated *Trichostrongylus colubriformis* whereby adult sheep attained a high level of immunity whereas vaccination of 3 month old lambs was less effective (Gregg *et al* 1978). However, vaccination with attenuated *T. circumcincta* was not as successful, with adult sheep only attaining slight resistance to homologous challenge (Smith *et al* 1982).

1.6.2 Surface and excretory / secretory antigens

Vaccine trials have been carried out using conventional surface or excretory / secretory (ES) antigens of parasitic nematodes. Jacobs *et al* (Jacobs *et al* 1999) vaccinated sheep with a *Haemonchus contortus* L3 surface antigen achieving up to 69% reduction in faecal egg counts following homologous challenge. Recombinant Cathepsin B and L, immunogenic virulence factors contained in the ES products of the liver fluke *Fasciola hepatica* (Dalton *et al* 2003) confer protection against

homologous challenge in rats (Jayaraj *et al* 2009) and cattle (Golden *et al* 2010). Ancylostoma secreted proteins (ASPs) and in particular ASP-2, found in the ES products of hookworms and associated with the transition to parasitism (Hawdon *et al* 1996), have demonstrated a protective capacity in recombinant form in dogs (Bethony *et al* 2005) and hamsters (Goud *et al* 2004; Mendez *et al* 2005), resulting in ASP-2 being investigated as a component of a human hookworm vaccine (Bethony *et al* 2008; Loukas *et al* 2006). Hc24, a *Haemonchus contortus* homologue of ASP-2, shows a protective capacity against homologous challenge in sheep in both native and recombinant forms, with a reduction in egg output of 32.2 % and 46% respectively, and a reduction in worm burdens of 64% and 65 % respectively (Schallig and van Leeuwen 1997; Vervelde *et al* 2002).

1.6.3 Gut membrane antigens

Substantial success has been attained in vaccine trials against the blood-sucking ovine nematode parasite *Haemonchus contortus* using glycoprotein antigens derived from the adult gut membrane, reviewed by Knox and Smith (Knox and Smith 2001) These protective gut membrane antigens include the aminopeptidase H11 (Munn *et al* 1997; Smith *et al* 1993), and the highly protective H-Gal-GP complex which includes aspartyl protease (pepsin), metalloproteases, cysteine protease, a thrombospondin homologue and a galectin (Knox and Smith 2001) and which leads to a reduction in worm burdens of 72% and reductions in faecal egg output of 93% (Smith *et al* 1994) following vaccination with native antigen. A

recombinant gut glycoprotein of the cattle tick *Boophilus microplus* has also been highly successful against homologous challenge in vaccine trials and is commercially available as TickGARD (Rand *et al* 1989; Willadsen *et al* 1995). The gut antigen approach has been tested for *T. circumcincta* and *O. ostertagi* but with limited success (Smith *et al* 2000; Smith *et al* 2001).

1.6.4 Vaccine trials against *T. circumcincta* and *O. ostertagi*

Vaccine trials in which sheep or cattle have been immunised against *T. circumcincta* or *O. ostertagi*, the antigen used and the degree of protection achieved, are listed in Table 1. Papers 4 and 5 in this thesis detail vaccine trials carried out against *Ostertagia ostertagi* and *Trichostrongylus axei* in cattle and sheep respectively, using detergent soluble antigens prepared from fourth stage larvae.

Table 1. Vaccine trials carried out against *T. circumcincta* in sheep and *O. ostertagi* in cattle.

All antigens derived from homologous species unless otherwise stated.

FEC, faecal egg counts. IM, intramuscular. SC, subcutaneous. IP, intraperitoneal. Bh, beryllium hydroxide. Al(OH)₃, aluminium hydroxide.

Negative numbers indicate an increase in faecal egg counts / worm burdens.

Antigen	Life stage	Administration	% protection	Reference
<i>Vaccine trials against Teladorsagia circumcincta</i> in sheep				
Gut membrane proteins	Adult <i>H. contortus</i>	IM + Quil A	1% FEC 0% worms	(Smith <i>et al</i> 2001)
Gut membrane proteins	Adult	IM + Quil A	28% FEC 8% worms	(Smith <i>et al</i> 2001)
Surface proteins	L3	SC + Bh	72 % worms	(Wedrychowicz <i>et al</i> 1995)
Oxfendazole abbreviated infection	Larval	Oral	88% FEC 97% worms	(Stankiewicz <i>et al</i> 1996)
Ivermectin abbreviated infection	Larval	Oral	5% FEC 22% worms	(Stankiewicz <i>et al</i> 1996)
31kDa antigen	L3	SC + Quil A	58% worms	(McGillivray <i>et al</i> 1992)
31kDa antigen	L3	SC + Quil A	23% worms	(Morton <i>et al</i> 1995)
31kDa antigen	L3	SC + Quil A	14% worms	(Morton <i>et al</i> 1995)

Antigen	Life stage	Administration	% protection	Reference
31kDa antigen	L3	SC + Quil A	38% worms	(Morton <i>et al</i> 1995)
Irradiated larvae (immunising infection not cleared before challenge)	L3	Oral	64% FEC 51% worms	(Smith <i>et al</i> 1982)
Irradiated larvae (immunising infection not cleared before challenge)	L3 <i>H. contortus</i>	Oral	68% FEC 47% worms	(Smith <i>et al</i> 1982)
Irradiated larvae (Immunising infection cleared before challenge)	L3	Oral	32% FEC 78% worms	(Smith <i>et al</i> 1982)
Lyophilised larval metabolites	L4 and early L5	IM	25% FEC 3% worms	(Rose 1978)
Lyophilised larval metabolites	L4 and early L5	IM + FCA	77% FEC 7% worms	(Rose 1978)
Macerated larvae	L4 and early L5	IM + FCA	44% FEC 66% worms	(Rose 1978)
Concentrated larval metabolites	L4 and early L5	IM	47% FEC 48% worms	(Rose 1978)
Concentrated larval metabolites	L4 and early L5	IM + FCA	57% FEC 60% worms	(Rose 1978)
Larval culture medium	L4 and early L5	IM + FCA	55% FEC 69% worms	(Rose 1978)

Antigen	Life stage	Administration	% protection	Reference
<i>Vaccine trials against Ostertagia ostertagi in cattle</i>				
ConA lectin binding glycoproteins (gp)	Adult	IM + Quil A	50% FEC	(Smith <i>et al</i> 2000)
Peanut lectin binding gp	Adult	IM + Quil A	23% FEC 11% worms	(Smith <i>et al</i> 2000)
ConA minus peanut lectin binding gp	Adult	IM + Quil A	28% FEC 24% worms	(Smith <i>et al</i> 2000)
ConA plus peanut lectin binding gp	Adult	IM + Quil A	30% FEC	(Smith <i>et al</i> 2000)
ES	L4	IP + FCA	11% FEC 6% worms	(Hilderson <i>et al</i> 1995)
Somatic Fraction 1	L4	IP + FCA	12% FEC 6% worms	(Hilderson <i>et al</i> 1995)
Somatic Fraction 2	L4	IP + FCA	6% FEC 5% worms	(Hilderson <i>et al</i> 1995)
Gut homogenate	Adult <i>H. placei</i>	SC + dextran sulfate	38% worms	(Siefker and Rickard 2000)
ES	L3 and L4	IP	3 6% worms	(Herlich and Douvres 1979)
S3 thiol	Adult	IM + Quil A	7% FEC 0% worms	(Geldhof <i>et al</i> 2002)
ES thiol	Adult	IM + Quil A	60% FEC 19% worms	(Geldhof <i>et al</i> 2002)
ES thiol	Adult	IM + Quil A	56% FEC 12% worms	(Geldhof <i>et al</i> 2004)
ES thiol	Adult	IM + Al(OH) ₃	7% FEC 31% worms	(Geldhof <i>et al</i> 2004)

Antigen	Life stage	Administration	% protection	Reference
ES thiol	Adult	IM + Quil A	62% FEC 6% worms	(Meyvis <i>et al</i> 2007)
ES thiol ASP enriched	Adult	IM + Quil A	74% FEC 47% worms	(Meyvis <i>et al</i> 2007)
ES thiol cysteine protease enriched	Adult	IM + Quil A	80% FEC 34% worms	(Meyvis <i>et al</i> 2007)
ES thiol – ASP/cysteine proteases	Adult	IM + Quil A	70% FEC 7% worms	(Meyvis <i>et al</i> 2007)
Polypeptide allergen (OPA)	L3 + L4 + Adult	IM + Quil A	59% FEC 2% worms	(Vercauteren <i>et al</i> 2004)
Recombinant OPA		IM + Quil A	24% FEC 14% worms	(Vercauteren <i>et al</i> 2004)
Globin	Adult	IP + FCA/FIA	52% FEC 28% worms	(Claerebout <i>et al</i> 2005a)
Globin	Adult	IM + Quil A	63% FEC	(Claerebout <i>et al</i> 2005a)
Globin	Adult	IM + Quil A	42% FEC	(Claerebout <i>et al</i> 2005a)
Globin	Adult	IM + Quil A	23% FEC	(Claerebout <i>et al</i> 2005a)
Aspartyl protease inhibitor (API)	Adult	IM + Quil A	109% FEC 44% worms	(De Maere <i>et al</i> 2005)
Recombinant API		IM + Quil A	76% FEC 37% worms	(De Maere <i>et al</i> 2005)
Recombinant Heat Shock Protein 18		IM + Quil A	12% FEC 38% worms	(Vercauteren <i>et al</i> 2006)
Recombinant ASP		IM + Quil A	7% FEC 24% worms	(Geldhof <i>et al</i> 2008)

1.7 Methodological Overview

Detailed materials and methods for each chapter are contained within each published paper. Brief overviews of the methods employed in the thesis are contained in this section.

1.7.1 Experimentally induced immunity model

The experiments involving lymphatic cannulation, described in papers 1, 2 and 3, used groups of sheep which had been immunised against *Teladorsagia circumcincta* via controlled trickle infection. This involved oral-dosing the sheep with infective larvae 3 times per week as represented in figure 1.3. Figure 1.3 also shows the timing of surgery, the challenge dose of larvae and the duration of lymph collection for both the immune and naïve groups of sheep involved in these experiments. The immune group of sheep described in paper 6 were immunised using the same trickle infection regime.

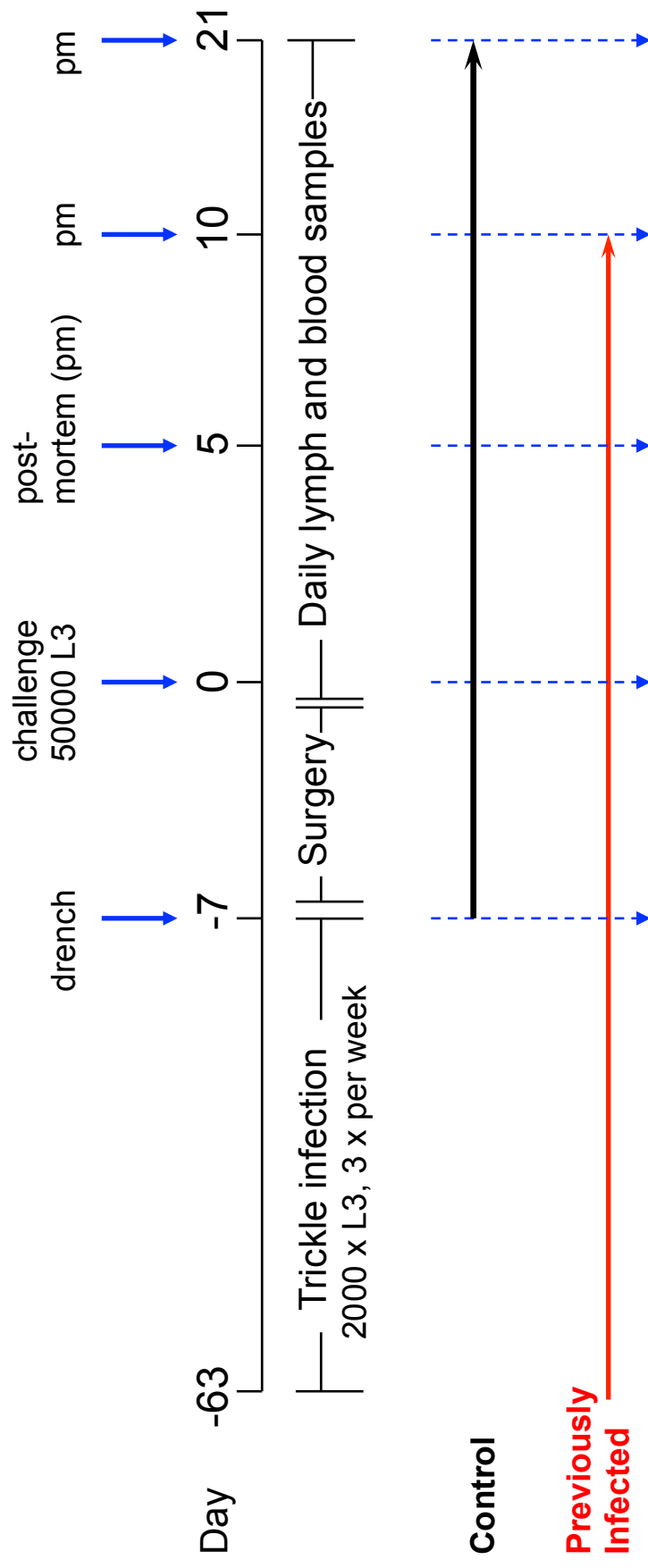


Figure 1.3 Experimental infection model.

1.7.2 Lymph duct cannulation

Cannulation of the efferent gastric lymph duct is a surgical procedure involving insertion of an in-dwelling cannula into the lymph duct, which is approximately 2mm in diameter. Briefly a surgical incision was made under general anaesthesia in the left side of the sheep caudal to the last rib. The abomasum was manipulated to the site of incision and evans-blue dye injected into the afferent lymphatic visible on the external surface. The abomasum was then returned to the abomasal cavity, and the liver held back by a surgical retractor to allow visualisation of the draining efferent gastric lymph duct containing the draining dye as shown in figure 1.4.

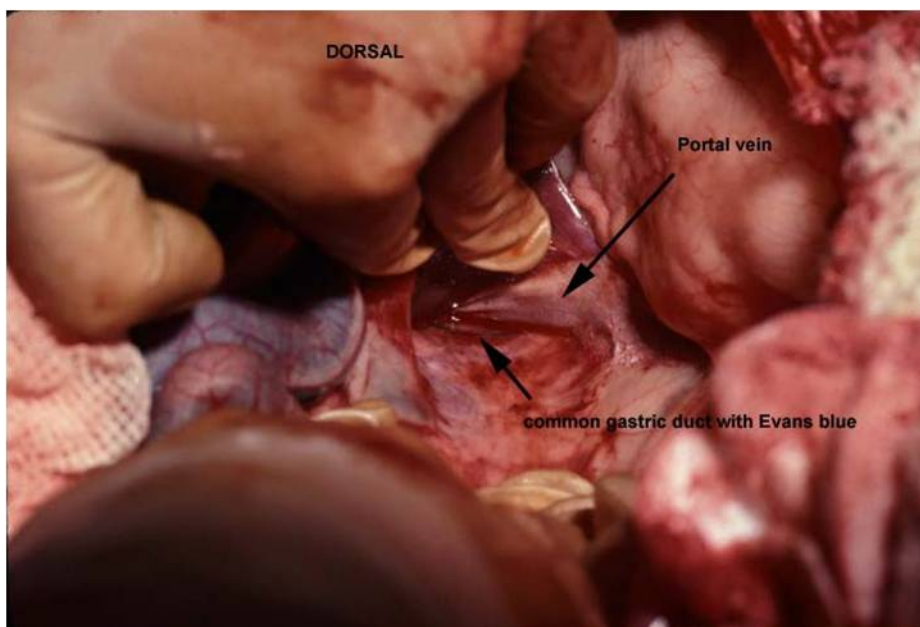


Figure 1.4. Visualising the efferent gastric lymph duct during cannulation surgery.

The lymph duct was tied off with Vicryl suture downstream of the proposed cannula insertion site to allow pooling of the lymph for better visualisation of the duct. A small hole was then cut in the lymph duct and the cannula inserted pointing upstream, after which the cannula was secured to the duct with a further length of suture.

A further cannula was inserted into the vena cava for the purpose of re-infusing collected lymph, then the surgical wound was closed and both cannulae secured onto the outer surface of the skin. The sheep were given antibiotics and painkillers and brought round from anaesthesia.

The sheep appeared to suffer no ill effects following surgery, regaining their mobility and appetite very quickly.

1.7.3 Lymph collection

Lymph was collected via the externalised cannula into sterile urine drainage bags containing heparin. A fresh subsample of approximately 20 ml was collected at 24 hour intervals into a sterile tube, before weighing and re-infusing the lymph collected in the drainage bag over the previous 24 hours. Blood samples were also collected on a daily basis.

1.7.4 Analysis of cellular and humoral responses

Cell counts to determine the numbers of normal and large basophilic cells in the lymph were carried out on a Coulter Counter. Cells were then stained with a panel of monoclonal antibodies against specific cell surface markers, fluorescently labelled with a phycoerythrin-conjugated secondary antibody, and analysed on a fluorescent activated cell sorting (FACS) machine. FACS analysis was carried out immediately following cell labelling, as storage at 4°C for even a short period of time resulted in signal deterioration. Humoral responses were analysed by enzyme linked immunosorbent assays (ELISA). A sandwich ELISA, where anti-IgA antibody was used both to coat the plates and as detection antibody, was used to measure total IgA levels, while plates were coated with parasite antigen then anti-IgA used for detection to measure levels of parasite specific antibody.

1.7.5 Parasitological techniques

Faecal egg counts were carried out using the modified McMaster technique (Baldock *et al* 1990). Worm burdens were enumerated post-mortem from both the abomasal contents and the abomasal mucus. Larvae were extracted from the abomasal mucus by suspending the abomasum lumen side down on polystyrene floating in warm saline solution in a Baermann funnel, which stimulated the larvae to wriggle out of the gastric glands and settle at the bottom of the funnel.

This was carried out in a designated room with room temperature set at 37°C.

Larval length was determined by tracing the larvae using a camera lucida at 50x magnification then measuring the length of the tracing.

1.7.6 Statistical analyses

Geometric means are presented for worm burdens, faecal egg counts, larval length, percentage of larvae arrested at the early L4 stage of development, antibody levels and cell counts. Two-tailed student's *t*-tests and one way analysis of variance (ANOVA) were used as appropriate to detect statistical differences between groups. A repeated measures model was used to analyse sequential measurements of antibody levels and cell counts. A Generalised Linear Model and Fishers Exact Test were used to detect statistically different levels of transcript abundance, Gene Ontology annotations and KEGG pathway representation in paper 6.

1.7.7 Antigen preparation for vaccine trials

Antigen for the vaccine trials described in papers 4 and 5 was prepared from fourth stage *O. ostertagi* and *T. circumcincta* respectively. Briefly sequential extracts were made of the larvae, in phosphate buffered saline (PBS), PBS-Tween then PBS-Triton. Affinity chromatography was carried out by passing the

PBS-Triton extract over a ConA lectin column. The bound material was eluted using the appropriate sugar solution and used as antigen for the vaccine trials.

1.7.8 Design of vaccine trials

Worm free cattle and sheep were injected intramuscularly with either antigen plus Quil A as adjuvant, or Quil A alone, 3 times at 3 week intervals. Oral larval challenge was carried out at the same time as the third vaccination, then egg counts monitored from approximately 18 days post challenge. The animals were slaughtered 35 days after challenge and worm burdens enumerated. Blood samples were taken at regular intervals for antibody analysis.

1.7.9 Generation of immune and naïve abomasal environments

Immune sheep were generated as detailed in figure 3. Following anthelmintic drench the immune sheep were given a bolus dose of 50,000 infective *T. circumcincta* L3, then these sheep and a group of parasite naïve sheep were slaughtered 24 hours later. Folds of abomasum were removed and incubated for 2 hours in Earles Buffered Saline Solution (EBSS). The abomasal folds were then removed and the buffer retained as either immune or naïve abomasal environment fluid.

1.7.10 Larval exsheathing and exposure to abomasal environment

Third stage *T. circumcincta* larvae were exsheathed by incubation for 4 hours in low pH EBSS at 37°C, with shaking, in an attempt to mimic a more physiological exsheathing environment compared to the standard method of chemical exsheathing in sodium hypochlorite. After 4 hours the larvae were transferred into either immune or naïve abomasal environment fluid for a further 4 hours.

1.7.11 Larval RNA extraction and cDNA preparation

Following exposure to the abomasal environment fluid, *T. circumcincta* larvae were snap frozen in liquid nitrogen, then RNA extracted using Trizol as per the manufacturer's instructions. cDNA was generated using the SMARTer™ cDNA synthesis kit from Clontech/TakaraBio. An optimised cDNA amplification protocol was used to avoid over cycling the PCR prior to transcriptome sequencing.

1.7.12 454 sequencing

Roche-454 sequencing was carried out by the GenePool, Edinburgh University, on the cDNA prepared from immune- and naïve- environment exposed *T. circumcincta* larvae.

1.7.13 Bioinformatic analysis

Bioinformatic analysis of the transcriptome sequencing data generated by The GenePool was carried out using a combination of commercially available resources (eg Roche Newbler assembly software (Roche 2010), freely available software (eg BLAST (Altschul *et al* 1997), UCLUST (Edgar 2010)) and in-house software developed specifically for the dataset by GenePool bioinformaticians (eg script for determining read count abundance of Clusters).

1.8 Aims

Knowledge regarding the immune response of sheep and cows to infection with *Teladorsagia circumcincta* and *Ostertagia ostertagi* respectively has increased substantially in recent years, however the exact mechanisms leading to protective immunity and the complex nature of the host-parasite relationship are not fully understood. Attempts to vaccinate sheep and cattle against these parasites have not achieved the level of success observed in vaccine trials against other nematode parasites. The aims of this thesis were therefore to:

1. Carry out a series of experiments involving cannulating the efferent gastric lymph duct of sheep and to use up-to-date molecular and immunological techniques order to monitor the local immune responses following *Teladorsagia circumcincta* infection of these sheep.

2. Assess whether any qualitative or quantitative differences could be detected between the immune responses of adult sheep and lambs following infection with *Teladorsagia circumcincta*.

3. Carry out vaccine trials against *Teladorsagia circumcincta* in sheep and *Ostertagia ostertagi* in cattle using antigens derived from 4th stage larvae.

4. Use modern transcriptomic sequencing technology and bioinformatics approaches to investigate the immediate responses of *Teladorsagia circumcincta* upon encountering ovine hosts of either naïve or immune status.

2 Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep.

Aileen M Halliday, Caroline R. Routledge, Stuart K Smith, Jacqui B Matthews and W. David Smith. (2007). *Parasite Immunol*, **29**: 425-434.

This is the first in a series of three papers revisiting the gastric lymph duct cannulation model, developed at Moredun Research Institute in the 1980s, to gain a more thorough understanding of the components and kinetics of the local immune response to infection with *Teladorsagia circumcincta*. Previous studies using this model had detected an increase in IgA in the gastric lymph of immune sheep after challenge (Smith *et al* 1983a). The IgA response of immune sheep after challenge with *Teladorsagia circumcincta* shows an association with reduced adult worm length and reduced fecundity of adult female parasites (Stear *et al* 1995b). These data suggest an important role for IgA in the host response against challenge infection, therefore the experiments described in the paper in this chapter were designed to provide greater insight into the timing and role of the IgA response.

The aim of this work was to monitor both total and parasite-specific IgA in the efferent gastric lymph of adult sheep following primary and challenge infection with *Teladorsagia circumcincta*, using modern techniques such as Enzyme Linked Immunosorbent Assay (ELISA), which allows for greater accuracy and more flexibility in determining the concentration of proteins in a sample. A novel method of 'equivalent age' calculation was developed in order to better

determine when worm stunting may have occurred, and how this related to the onset of the IgA response in individual sheep.

Contribution to the work

The candidate was involved in surgery to cannulate the gastric lymph ducts, carried out post-mortem procedures and parasitological analyses, was part of a team collecting and processing daily lymph and blood samples for the duration of the experiment, was involved in all stages of experimental design, conduct and analysis, including antigen preparation, protein purification and ELISA. Results were presented at local, national and international conferences and meetings.

Parasite loss and inhibited development of *Teladorsagia circumcincta* in relation to the kinetics of the local IgA response in sheep

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SUMMARY

Groups of yearling sheep, which had been trickle infected with *Teladorsagia circumcincta* for 8 weeks and then drenched, were challenged with 50 000 *T. circumcincta* larvae together with groups of worm-free controls. Fewer parasites and a greater proportion of early fourth stage larvae were recovered from previously infected sheep compared to controls. Worm loss and arrested development were evident by 5 days after challenge whereas growth retardation of developing worms was observed by day 10. In the previously infected sheep a secondary IgA response was observed in the efferent gastric lymph from 5 days post-infection. Western blot analysis showed the lymph IgA to be predominantly dimeric and nonsecretory in nature and that the somatic antigens recognized were predominantly in the 100–250 kDa range. The concentration of IgA in lymph was always higher than in blood and in the previously infected sheep increased fivefold 8 days post-challenge in contrast to blood where IgA levels were unchanged. The timing of the response suggested that it occurred too late to have been the cause of worm loss or arrested development, though it may have retarded the growth of developing parasites.

Keywords acquired immunity, gastric lymph, IgA, *Teladorsagia circumcincta*

INTRODUCTION

Teladorsagia circumcincta is a parasitic nematode which inhabits the abomasum of sheep. It is well recognized as an important parasite in temperate regions, both in terms of animal welfare and productivity. Current control methods rely on the use of anthelmintics; however, resistance to these drugs is on the increase and there is evidence of isolates which exhibit phenotypic resistance to several classes of anthelmintic (1,2).

Sheep infected with *T. circumcincta* can acquire protective immunity (3–6), so vaccination may be a viable alternative control method (7–10). Manifestations of immunity to *T. circumcincta* include exclusion or expulsion of incoming third stage larvae (L3), inhibited development at the early fourth stage (EL4), retardation of adult worm growth and reduced fecundity (3,11,12). The success of adoptive transfer experiments in which immunoblast cells from immune sheep responding to infection were transferred to genetically identical, naïve sheep indicated that these anti-parasite effects are under immunological control (13). However, the exact effector mechanisms responsible for protection remain to be elucidated. Possible roles for both immediate hypersensitivity reactions and antibody responses in regulating parasite burden and length have been proposed: IgA responses have been associated with reductions in adult worm length (9–11,14,15), whereas IgE responses have been correlated with reduced faecal egg counts in grazing lambs (16,17). Here, we present further evidence that sheep which have been previously infected with *T. circumcincta* do acquire immunity to challenge infection and describe the nature and timing of the ensuing local IgA response.

MATERIALS AND METHODS

Sheep

Two experiments were conducted involving a total of 46 Scottish Blackface-cross yearlings. These had been reared

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under conditions designed to exclude accidental infection with nematode parasites.

Infective larvae

Infective L3 were from an anthelmintic susceptible *T. circumcincta* isolate which had been passaged through sheep at Moredun Research Institute for a number of years. The L3 were stored at 4°C for up to a month before administration. All challenge doses used within each experiment were derived from the same batch of larvae.

Surgical procedures

The common gastric lymph duct, which contains efferent lymph draining all four stomachs, was cannulated as detailed elsewhere (18). The sheep were fitted with an indwelling venous catheter placed in the jugular vein or the posterior vena cava.

Collection, sampling and re-infusion of lymph

Cannulated sheep were housed individually in small pens and lymph was collected into sterile, heparinized urine drainage bags supported by a harness as described before (19). Every morning the bags were weighed to estimate the lymph flow rate, and lymph and blood samples (10–20 mL) were collected into heparinized tubes. The bag contents were then re-infused intravenously under gravity.

Post mortem procedures

At the end of each experiment the sheep were stunned with a captive bolt, exsanguinated, and the abomasum removed and opened along its lesser curvature. The contents were collected and the abomasum was washed in two changes of warm saline. These washings were added to the contents. The abomasum was then placed in saline for 4 h at 37°C as described previously to recover larvae in the mucosa (20). These abomasal digests and the abomasal contents were made up separately to 5 L and fixed in 2% formalin.

An approximate 5% aliquot of each was obtained by stirring vigorously and sampling with a ladle. The exact volume of the subsample was then measured.

Worm counting, measuring and staging

Each subsample was stained by the addition of a concentrated iodine solution. The number of male, female and sexually undifferentiated EL4 was recorded. A random sample of approximately 50 parasites was obtained from each animal and these were measured by a camera Lucida

under $\times 10$ magnification. Sexually undifferentiated worms measuring < 1.5 mm were classified as EL4, longer parasites were designated 'developing worms'.

Calculating 'equivalent age' of developing worms

It was assumed from the work of Denham (21) that the *T. circumcincta* worms in the control sheep in the current experiment grew at a linear rate (see also Figure 2). Therefore linear regression of the mean lengths of the parasites recovered on days 5 and 10 was used to estimate the growth rates of each sex. The resulting equations were used to calculate the 'equivalent age' of the developing worms recovered from each previously infected sheep.

Statistical methods

Arithmetic means with standard errors are shown throughout. Parasite counts and percentage EL4 were compared by student's *t*-test. Frequency distributions of male and female worm lengths were made for individual sheep and group mean distributions were calculated from these. Immunoglobulin and antibody concentrations were compared using students *t*-test, and, after log transformation, by repeated measure models (Genstat). Correlations between worm burdens and antibody titres were carried out using Pearson's correlations on ranked data. $P < 0.05$ is regarded as the significant level of probability throughout.

Design of experiments

Both experiments compared the number and length of worms recovered from previously infected or control naïve sheep killed between 5 and 21 days following a single challenge dose of 50 000 *T. circumcincta* larvae (Table 1). The previously infected groups had received a trickle immunizing infection of 2000 infective L3 three times per week for 2 months, which was cleared out with anthelmintic before challenge as detailed in Table 1. Gastric lymph was obtained for more than a week from eight previously infected and eight control sheep.

Preparation of *T. circumcincta* L4 somatic and ES antigens

Helminth-free sheep were infected orally with 50 000 L3 and mucosal L4 recovered 7 days later using previously published methods (22). L4 parasites were homogenized in 2 mL ribolyser tubes (Q-BIOgene Lysing matrix D ceramic beads, Irvine, CA, USA) for 45 s in PBS containing 1 mM EDTA and 1 mM PMSF, then the homogenate centrifuged at 20 000 *g* for 20 min. The supernatant, containing L4 somatic extract, was retained and the protein concentration estimated. ES proteins were prepared as described previously (7).

Table 1 Design of experiments

Experiment	Group	Treatments (relative to day of challenge)						
		Trickle infection ^a	Levamisole (5 mg/kg)	Cannulate gastric lymph duct	Challenge infection ^b	Number killed		
		(-62 to -9)	(-7)	(-6 to -2)	(0)	(5)	(10)	(21)
1	Previously infected	+	+	+	+	–	6	–
	Control	–	+	+	+	–	6	4
2	Previously infected	+	+	+	+	6	6	–
	Control	–	+	+	+	6	6	6

a, 2000 *T. circumcincta* L3 3 times per week for 8 weeks; b, $1 \times 50\,000$ *T. circumcincta* L3.

Purification of ovine secretory IgA (sIgA)

IgA, for use as a standard in the quantification assay, was purified from lung fluid obtained from a sheep with pulmonary adenomatosis, an excellent source of this immunoglobulin isotype as described previously (23). The lung fluid was diluted 1 : 3 in PBS, pH 7.4, and loaded onto a 1 mL HiTrap Protein G column (Amersham Biosciences, Buckinghamshire, UK) pre-equilibrated with PBS. The column was washed with 4 volumes of PBS and the unbound material collected. Unbound protein, enriched for IgA and depleted of IgG, was concentrated to 250 µL at 4°C using centricon 10 filter devices (Amicon, Millipore, Billerica, MA, USA) and subsequently passed through a superose 12 column (Amersham Biosciences) coupled to a FPLC apparatus at a flow rate of 0.3 mL/min. The 500 µL fractions that were collected were analysed by SDS-PAGE and Western blotting using a mouse anti-bovine/ovine α chain monoclonal antibody (Serotec MCA628 AbD Serotec, Morphosys, Martinsried/Planegg, Germany). Fractions eluted early in the separation procedure which were found to contain only sIgA were pooled (see also Figure 3a, lane i), and their protein concentration estimated using the BCA kit (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

SDS-PAGE

Prior to SDS-PAGE samples were heated at 100°C for 3 min in an equal volume of 63 mM Tris-HCl pH 6.8 containing 5% (w/v) SDS, ± 10 mM DTT under nonreducing or reducing (10 mM DTT) conditions and separated on 4%–12% gradient acrylamide gels (Biorad, Hercules, CA, USA). Molecular weight markers (Fermentas, Burlington, ON, Canada) were run on each gel and the gels were stained with Coomassie Blue.

Western blotting

SDS-PAGE separated proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA) using a semidry apparatus (Sigma). Membranes were blocked in 10% Infasoy (Cow & Gate, Trowbridge, Wiltshire, UK) in 10 mM Tris, 0.5 M NaCl, 0.05% (v/v) Tween-20, 0.02% (w/v) thimerosal (TNTT), the assay diluent and wash buffer, for 1 h at room temperature, washed and incubated with the antibody reagents as specified below. After a further wash, the blot was developed using diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA) substrate and the reaction stopped after 2 min by washing in several changes of distilled water.

For characterization of its IgA structure, lymph was first depleted of albumin and IgG using commercially available spin columns (Pierce ProteomeLab) and the membranes were probed with polyclonal mouse anti-ovine IgA horseradish peroxidase conjugated antibody (Serotec, Raleigh, CA, USA AHP949P, diluted 1 : 1000) for 1 h at room temperature.

For analysis of the specificity of lymph IgA antibodies, blots of L4 somatic antigen were incubated with pooled lymph samples (1 : 20) from either naïve or previously infected sheep, collected 0 or 8 days post-challenge, then incubated sequentially with monoclonal mouse anti-ovine IgA horseradish peroxidase conjugated antibody (SERO-TEC, MCA628, 1 : 250) and polyclonal anti-mouse-HRP (Dako, Glostrup, Germany P0260, 1 : 1000).

Estimation of antibody titres: total IgA and L4 – specific IgA

Microtitre plates were coated overnight at 4°C with 50 µL coating protein anti-ovine IgA (Serotec, MCA628), L4 somatic extract or ES product at 5 µg/mL in 50 mM bicarbonate buffer, pH 9.6. The plates were washed six times with wash buffer (PBS, 0.05% v/v Tween-20), then incubated with

5% (v/v) horse serum in TNTT overnight at 4°C. After washing, 50 µL gastric lymph, diluted 1 : 16 000, or blood plasma, diluted 1 : 1000, were added for 2 h at room temperature. The wells were re-washed and 50 µL secondary antibody added for 2 h at room temperature (biotinylated monoclonal anti-ovine IgA diluted 1 : 100, or monoclonal anti-ovine IgA without biotin diluted 1 : 250). After a further wash, 50 µL of horseradish peroxidase-conjugated streptavidin (DakoCytomation, Glostrup, Germany) or polyclonal anti-mouse immunoglobulin (Dako, P0260, 1 : 500) were added for 1 h at room temperature. After a final wash, 50 µL *o*-phenylenediamine dihydrochloride substrate (Sigma) were added to each well. After 20 min in the dark, the colour reaction was stopped by addition of 25 µL 2.5 M sulphuric acid and OD values read at 490 nm. Each test sample was assayed in triplicate. For estimation of total IgA, serial dilutions of purified IgA (10–0.15 ng/mL) were included in triplicate on each plate to provide a standard curve from which the concentration of IgA in each gastric lymph or blood sample was calculated. For estimation of relative amounts of antibody, lymph from a previously infected sheep with a high parasite-specific antibody titre was included on each plate as a reference sample.

RESULTS

Parasitological data

Both experiments shared the same design (Table 1), their main purpose being to determine whether the anti-parasite effects could be correlated with the magnitude and/or the kinetics of the responses detected in gastric lymph, specifically

here the IgA response. The worm burdens and percent EL4 data were similar in the two separate experiments (Figure 1). Within each experiment, parasite counts in control animals were lower and more variable on day 21 than on day 10 post-challenge ($P < 0.01$, Figure 1a). Comparison between the previously infected and control groups 10 days after challenge showed that there were significantly ($P < 0.01$) fewer parasites in the previously infected sheep. In addition, data from experiment 2 showed that worm loss observed in the previously infected sheep had occurred by day 5 post-challenge (Figure 1a). Almost all parasites from sheep killed 5 days post-challenge were recovered from the abomasal digests, that is, they were present in the mucosa, as would be expected for early fourth stage *Teladorsagia*.

Less than 10% of the worms recovered from the control sheep at 5, 10 or 21 days post-infection were EL4 (Figure 1b). The percentage EL4 in the previously infected group was considerably higher on days 5 and 10 than in challenge control animals, but there was wide individual variation and so the differences between the groups were not statistically significant. In each experiment, developing worms (i.e. those longer than 1.5 mm) recovered from the previously infected sheep tended to be shorter than those obtained from the control animals. Pooling the data from the two experiments revealed that this difference in length was statistically significant ($P < 0.01$) for both male and female parasites (Figure 1c).

The mean lengths of the male and female developing *T. circumcincta* recovered from individual control sheep 5 or 10 days after challenge are plotted in Figure 2. The values obtained were slightly smaller than those recorded by Denham (21), possibly due to differences in measuring

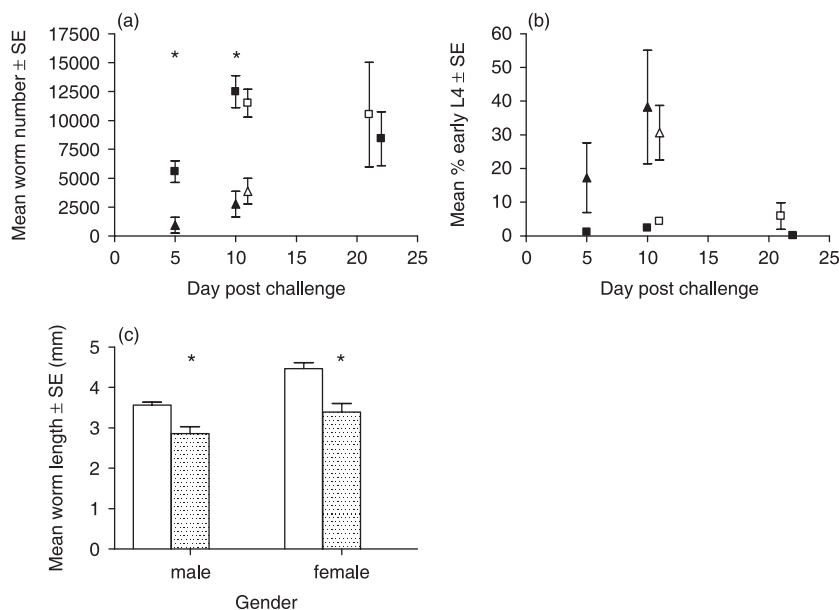


Figure 1 Parasitology data.

Squares = controls; triangles = previously infected; open symbols = Experiment 1; closed symbols = Experiment 2; * = ($P < 0.01$). (a) Total worm numbers, (b) percent early fourth stage larvae recovered. (c) Mean lengths of male and female worms recovered 10 days post-challenge. White bars = controls. Grey bars = previously infected.

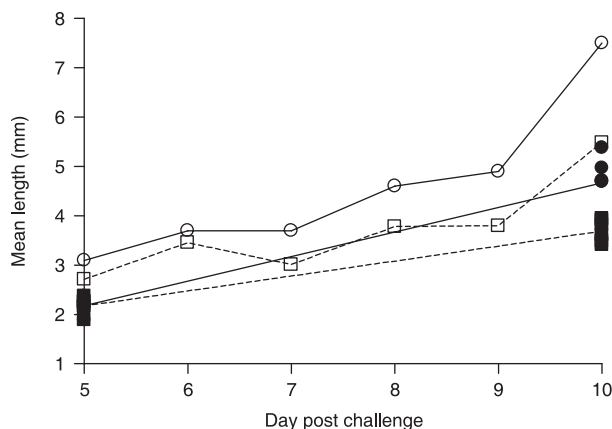


Figure 2 Growth rates of fourth stage *T. circumcincta*. Circles connected by solid lines = female parasites; squares connected by broken lines = males; open symbols = data extracted from Denham (21); closed symbols = mean length of worms recovered from individual control sheep in Experiment 2, connected by their respective regression lines.

technique, type of sheep, isolate of parasite or a combination of these factors. Linear regression yielded $y = 0.498 (\pm 0.052) \times -0.309 (\pm 0.041)$ and $y = 0.303 (\pm 0.022) \times +0.662 (\pm 0.177)$, for female and male worms, respectively, where y = mean length (mm) and x = days after infection. The equivalent age of developing female parasites ranged from 5.2 to 9.8 days and that of males ranged from 4.5 to 10.8.

Lymph flow

Except for one animal, where lymph flow stopped on day 8 post-challenge, lymph was collected until day 21 from eight

control sheep. Flow was maintained until post mortem at day 10 post-challenge in six previously infected sheep, although data from single sheep in which flow stopped on days 6 and 8 are also included in the group mean calculations for the relevant time points. Mean lymph flow throughout the experiment was 18.6 ± 0.7 mL/h. No significant differences were observed in lymph flow rates between the treatment groups or between experiments. Because of this and the similarity of the parasitology results, lymph data from each treatment group were pooled irrespective of experiment.

Structural characteristics of IgA in gastric lymph

Western blot analysis of IgA in lymph and sIgA from ovine lung fluid was carried out using a polyclonal antibody which binds to the α chain and the secretory component of IgA. While both the heavy chain (~60 kDa) and the secretory component (~80 kDa) were detected in 'reduced' sIgA, only the heavy chain could be detected in lymph (Figure 3a, lane iii; 3b, lanes iii and iv). Under nonreducing conditions sIgA appeared as two high molecular weight multimers (Figure 3a, lane i; 3b, lane i), whereas most IgA in lymph was smaller (Figure 3b, lane i), probably due to the absence of the secretory component. A small amount of apparently monomeric IgA (mw ~200 kDa) was also detected in the lymph (Figure 3b, lane i).

Evidence that IgA in gastric lymph is locally produced

Paired samples of blood and lymph collected 0 and 8 days post-challenge from previously infected sheep were assayed for total IgA (Figure 4). IgA concentrations were always higher in lymph than blood. Furthermore, a five- to sixfold

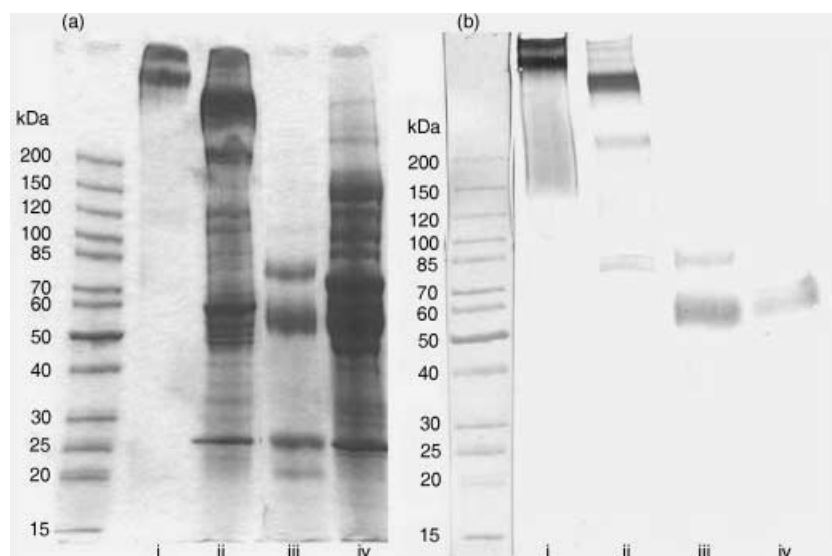


Figure 3 SDS-PAGE and Western blot analysis of IgA in lymph and secretory IgA (sIgA) from lung fluid. (a) SDS-PAGE. (b) Western blot. Lane i, sIgA nonreduced. Lane ii, lymph nonreduced. Lane iii, sIgA reduced. Lane iv, lymph reduced.

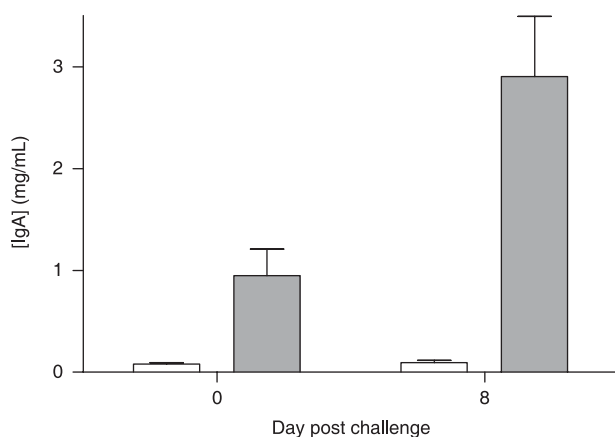


Figure 4 Total IgA in lymph (grey bars) and blood plasma (white bars) at days 0 and 8 post-challenge.

increase in lymph, but not blood, IgA concentrations was observed on day 8 post-challenge.

Changes in total IgA concentrations in gastric lymph following challenge

Mean total IgA levels in control sheep were slightly elevated compared to the previously infected sheep (1.6 mg/mL compared to 1 mg/mL) before challenge (Figure 5a). This difference was not significant and, at the time of infection, mean IgA levels in both groups were in the same range.

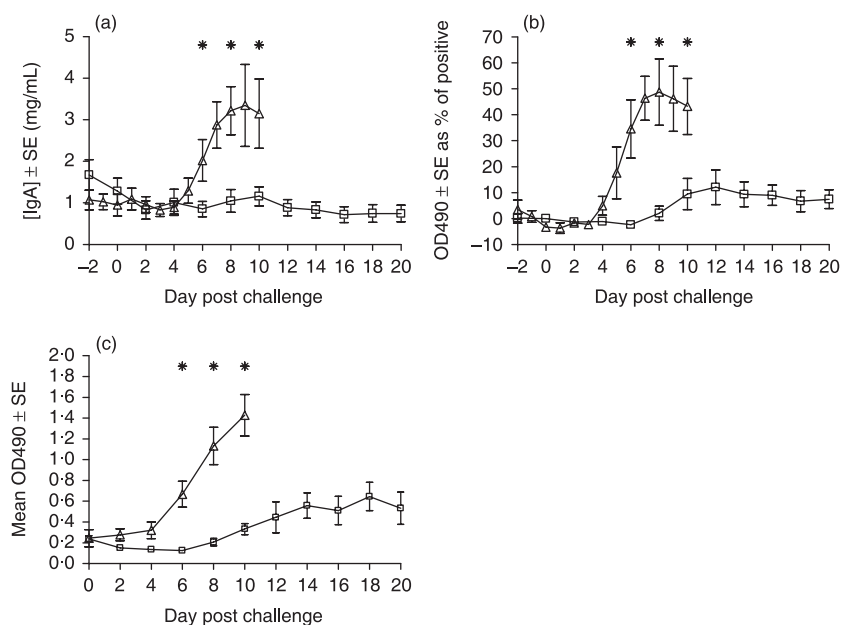


Figure 5 Mean gastric lymph IgA concentrations of control (squares) and previously infected (triangles) sheep. ** days when mean IgA concentrations of previously infected sheep were significantly ($P < 0.05$) higher than pre-challenge (day 0) or control values. (a) Total IgA. (b) *T. circumcincta* L4 somatic antigen specific IgA. (c) *T. circumcincta* L4 ES antigen specific IgA.

Control sheep showed no significant change in total IgA throughout the experiment. However, total IgA output in the previously infected group started increasing from 0.89 ± 0.16 mg/mL approximately 4 days post-infection, peaking at 3.35 ± 0.99 mg/mL 9 days post-infection. The differences between pre- and post-infection IgA levels were significant on days 6–10 post-challenge ($P < 0.05$). Standard curve data points for each plate showed a consistent linear response, with negligible background.

Teladorsagia circumcincta L4 somatic and E/S antigen-specific IgA responses in gastric lymph

Teladorsagia circumcincta L4 somatic antigen-specific IgA levels are shown in Figure 5b. Pre-challenge levels of antigen-specific IgA in both groups of sheep were in the same range. In parallel with the mean total IgA levels, control sheep showed no significant change throughout the experiment. However, compared to pre-challenge levels, the previously infected sheep showed an increase ($P < 0.05$) in somatic antigen-specific IgA from 5 days post-challenge, which rose to levels significantly ($P < 0.05$) higher than control sheep on 6, 8 and 10 days post-infection (Figure 5b).

Analysis of the anti-L4 ES responses also revealed a clear difference between the groups (Figure 5c), with the previously infected sheep showing an increase in specific IgA by 6 days post-challenge, with significantly ($P < 0.05$) higher IgA levels compared to both pre-challenge and control sheep values on days 6, 8 and 10.

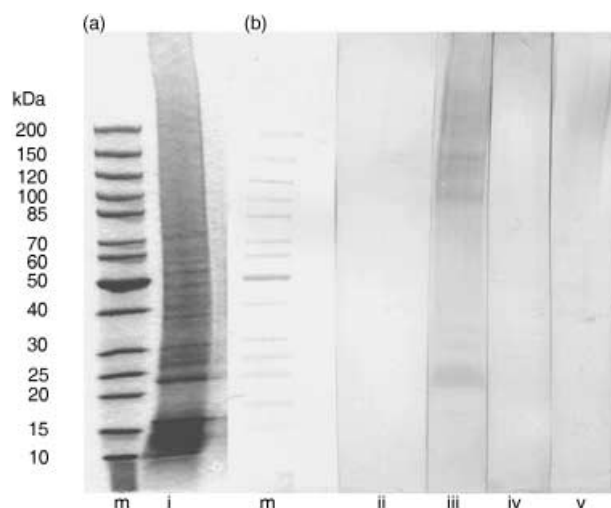


Figure 6 Specificity of gastric lymph IgA for *T. circumcincta* L4 somatic extract. Panel (a) shows an SDS-PAGE gel of the extract (lane i) and molecular weight markers (lane m) stained with Coomassie Blue. Panel (b) shows a blot of the same preparation probed with pooled lymph from previously infected sheep 0 (lane ii) or 8 (lane iii) days post-challenge, and pooled lymph from naïve sheep 0 (lane iv) or 8 (lane v) days post-challenge, compared to molecular weight markers (lane m).

Western blot analysis of L4 somatic antigen specific IgA response

Western blot analysis confirmed that several antigens from L4 somatic extract were recognized by gastric lymph IgA from previously infected sheep after challenge, with negligible IgA antibody activity against somatic antigens from either worm free or previously infected sheep prior to challenge (Figure 6). The antigens recognized ranged in size from approximately 20 kDa to over 200 kDa, with the most immunogenic predominantly ≥ 100 kDa.

Correlation between the manifestations of immunity and the local antigen-specific IgA response

No significant correlations were observed when the antigen-specific or total IgA responses of individual sheep were compared with the number of worms, or percentage EL4, obtained at necropsy. The equivalent age of the parasites recovered from seven individual previously infected sheep at 10 days post-challenge, were plotted beside their respective antigen-specific and total IgA responses (Figure 7). IgA was observed to rise before the 'equivalent age' of the developing worms was reached, but the response was too slow to be implicated in either parasite loss or arrestment, both of which had occurred by day 5.

DISCUSSION

In the two experiments described here, three manifestations of immunity to *T. circumcincta* were observed in the previously infected sheep: increased worm loss, an increase in the proportion of arrested EL4 and a reduction in the growth of developing parasites. Parasite loss, or failure of larvae to establish in the abomasal mucosa, is the most important manifestation of immunity. Parasite numbers in the previously infected sheep were significantly reduced by 5 days post-challenge. Previous studies, which utilized the infection model described here, indicated that substantial parasite loss occurs as early as 2 days post-challenge and is the result of an active immune response rather than a pre-existing passive state (12). The difference in parasite counts observed in sheep from both groups killed at days 5 and 10 post-challenge, with higher numbers obtained at the latter time point, probably reflects the relative inefficiency of recovering day 5 larvae from the gastric mucosa, and has been observed previously (13). The control sheep also appear to have lost a portion of their worm burden between days 10 and 21 post-challenge.

Arrestment of *T. circumcincta* at EL4 is a well recognized phenomenon (24–27). Here, the finding that arrestment was far more prevalent in the previously infected compared to the challenge control sheep indicates that this state occurs as the result of acquired immunity (26). Parasite factors, such as storage conditions, strain type and density-dependent effects, known to influence the degree of arrestment in this and other genera (28), can be ruled out here as all sheep received L3 from the same batch on the same day. Since the sheep were of similar age and from the same flock, potential host factors such as breed and maturity can also be largely excluded.

Significantly shorter developing worms were recovered from the previously infected sheep. Calculating their 'equivalent age' from the growth curve provided by worms recovered from the challenge control sheep, identified the time before which the cause of stunting was operating.

Uniquely, cannulation of the common efferent gastric lymph duct allows the kinetics of both cellular and humoral aspects of the local immune response to infection with abomasal nematodes to be monitored in individual sheep in a physiological manner over a period of weeks. The timing of the various components of this response can be correlated with the timing of the various manifestations of immunity to *Teladorsagia*. This paper focuses on the IgA component of that response.

In the ruminant the common gastric lymph duct drains all four stomachs. It does not receive lymph from the intestines (29). Furthermore, as the rumen, reticulum and omasum do not possess mucosal surfaces, IgA concentrations in the

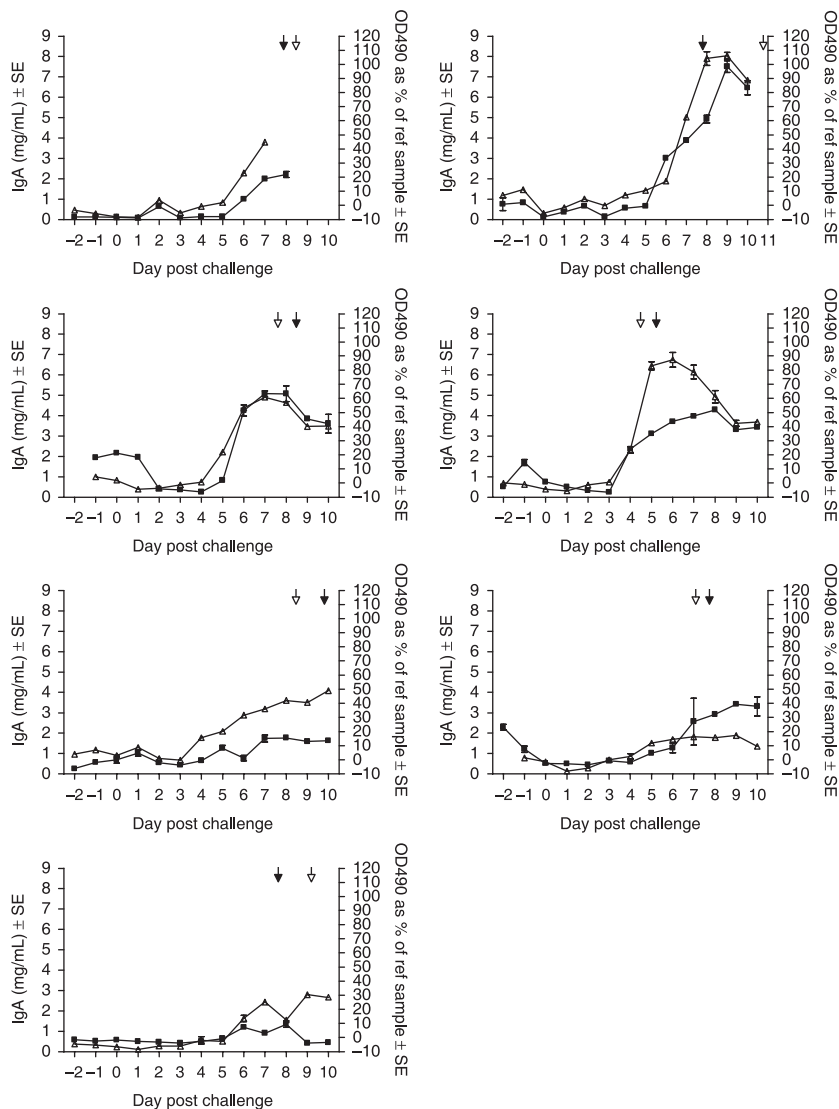


Figure 7 Equivalent age of developing worms recovered 10 days post-challenge from individual previously infected sheep. Total (left y-axis, mg/mL) and L4 somatic antigen specific (right y-axis, % of ref sample) IgA responses of individual previously infected sheep. Downwards arrows = mean equivalent age of developing worms, solid arrows = females, open arrows = males.

gastric lymph overwhelmingly reflect that of the interstitial fluid of the abomasal mucosa. Our unpublished findings show, that compared to the abomasal mucosa, the gastric nodes contain few IgA containing cells and contribute little IgA to the efferent lymph. This is supported by reports that IgA concentrations in afferent and efferent ovine intestinal lymph during nematode infection were very similar (30).

It could be argued that it would have been better to follow the IgA response in abomasal mucus, the environment in which it interacts with *Teladorsagia*. Unfortunately, sampling of true abomasal mucus is only possible *post mortem*, which rules out sequential measurements from the same individual. In addition, assaying abomasal IgA responses in *post mortem* mucosal scrapings suffers from the additional disadvantage of the samples containing a nonphysiological mixture of interstitial and mucus IgA.

Gel and Western blot analysis showed, for the first time to our knowledge, that ovine gastric lymph IgA was dimeric and lacked secretory component, as described for circulating IgA in other mammals (31). This is in line with the model whereby polymeric IgA binds covalently to the polymeric Ig receptor (pIgR) on the basolateral surface of mucosal epithelial cells, and is then transcytosed along with the pIgR to the apical membrane, whereupon the pIgR is cleaved releasing the IgA with part of the pIgR, the secretory component, still attached, into the mucosal secretions (31,32).

The mean peak daily output of lymph IgA was calculated (lymph flow rate \times IgA concentration) as 1.09 g/day, a substantial amount of protein. However, if the majority of the dimeric IgA produced in the mucosa is actively secreted into the abomasal mucous, this would only represent a

small fraction of the total local production of this isotype. It is also possible that due to the difference in molecular weights between the sIgA used as a standard in the ELISA and the dimeric IgA in the gastric lymph, the total IgA content of the gastric lymph may have been underestimated by up to 25%. The specificity of the IgA response to L4 ES products has been reported previously (7). In this paper gastric lymph IgA from immune sheep is also shown to recognize a range of antigens from the L4 somatic extract.

Clear anamnestic IgA responses were detected in the previously infected sheep from 5 days post-infection, which agrees with previous findings (3,12). By analysing immune responses in gastric lymph over time, it is possible to compare the kinetics of specific parameters (in this case IgA) with the timing of the various manifestations of immunity. Since parasite loss and arrested development had occurred by 5 days post-infection, that is, before IgA started to peak, it is unlikely that this isotype was the primary cause of these anti-parasite effects. However, the IgA response did occur sufficiently rapidly to be a possible cause of parasite stunting, although no correlation could be found between the magnitude of the individual response and the length of parasites observed in each sheep. Significant inverse correlations have been identified between the length of *Teladorsagia* recovered from naturally or deliberately infected sheep and the concentration of IgA found in mucosal scrapings from the abomasum (9,11,14). More recently, these findings have been extended to indicate that, in grazing lambs, circulating IgA levels are associated with the degree of larval arrestment (33); however, the findings reported here suggest that the IgA response does not occur quickly enough to be the immunological cause of inhibition.

An advantage of the experimental design employed here is that it produces a readily measurable degree of acquired immunity which is relatively reproducible (Figure 1). Anthelmintic treatment to remove any residual immunizing worms allows challenge parasites to be easily identified and analysed, but at the same time may cause a waning of local immune responses. For this reason, it is not clear how well the design represents what happens under natural conditions where sheep experience a continuous input of new larvae. After a few weeks of continuous exposure, newly acquired worms will arrive in the face of a pre-existing immune response (including high IgA concentrations). It may be premature therefore to conclude from the current data that IgA is not implicated in worm loss or arrestment.

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3 Kinetics of the local cellular response in the gastric lymph of immune and susceptible sheep to infection with *Teladorsagia circumcincta*.

Aileen M Halliday, W. Ivan Morrison and W. David Smith (2009). *Parasite Immunol*, **31**: 402-411.

This is the second in a series of 3 papers using the gastric lymph cannulation model developed at Moredun Research Institute to investigate the timing and components of the ovine immune response to infection with *Teladorsagia circumcincta*. Previous studies using this model detected rapid expansion of lymphocytes following challenge infection of previously infected sheep (Smith *et al* 1983a and Smith *et al* 1984). Subsequent experiments have revealed the expansion of parasite specific CD4+ cells and antibody secreting cells in the abomasal lymph nodes (Almeria *et al* 1998; Balic *et al* 2003; Gasbarre 1994). The aim of the experiments described herein was to use up-to-date immunological techniques such as FACS to further investigate the components of efferent gastric lymph and the timing of response in both parasite naïve and previously infected adult sheep, following exposure to *Teladorsagia circumcincta*.

Contribution to the work

The candidate was involved in all stages of experimental design and planning, was involved extensively in surgery to cannulate the gastric lymph ducts of sheep, was involved in daily collection of lymph and blood samples for the

duration of the experiment, and carried out cellular analysis and data processing and analysis. Results were presented at local, national and international meetings and conferences.

Kinetics of the local cellular response in the gastric lymph of immune and susceptible sheep to infection with *Teladorsagia circumcincta*

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SUMMARY

Groups of yearling sheep were trickle infected with *Teladorsagia circumcincta* for 8 weeks, then the infection cleared with anthelmintic and both these animals and a group of parasite naïve sheep were challenged with 50 000 infective *T. circumcincta* larvae. The previously infected sheep demonstrated acquired immunity to the parasite, manifested by reduced worm burdens which were evident as early as 2 days after challenge. Cannulation of the common efferent gastric lymph duct allowed the kinetics of their local cell traffic to be monitored, and the phenotype of these lymphocytes was analysed. A blast cell response, consisting of both T and B lymphocytes, was observed in both groups of sheep, however this occurred more rapidly in the previously infected, immune animals. CD4⁺, CD8⁺ and CD25⁺ blast cell output peaked at day 3 in the previously infected animals, whereas CD21⁺ blast cell output peaked slightly later at day 5. In the control group the peak output of all phenotypes of blast cells occurred more slowly, peaking 10 days after infection.

Keywords acquired immunity, cellular response, gastric lymph, lymphocyte subsets, sheep, *Teladorsagia circumcincta*

INTRODUCTION

Teladorsagia circumcincta is an abomasal nematode parasite of sheep, which is well recognized as an important pathogen in temperate regions, both in terms of animal welfare and economic loss. Current control methods rely on the use of anthelmintic drugs; however, parasite resistance to these is widespread and increasing, and there are now reports of isolates of *T. circumcincta* which are resistant to several classes of anthelmintic (1). As sheep can acquire immunity to *T. circumcincta* (2–5), vaccination may be an alternative prospect for future control (6–9); however, the exact effector mechanisms of immunity remain to be elucidated.

Immunity to *T. circumcincta* manifests itself in several ways, including worm loss, inhibited development of larvae at the early fourth stage (EL4), stunted growth of developing worms and reduced fecundity (10,11). Possible roles for both immediate hypersensitivity responses and antibodies have been proposed. For example, IgE responses have been correlated with reduced faecal egg counts in grazing lambs (12,13), whilst IgA, the main mucosal antibody isotype, has been associated with reduced adult worm length and fecundity (10,11,14,15). In the experimental infection model employed here, the secondary IgA response observed in the gastric lymph of immune animals has been shown to occur rapidly enough to be involved in the stunting of developing worms, but too slowly to be responsible for worm loss or arrestment at the early L4 stage (11). The cellular immune response has also been demonstrated to be an important aspect of acquired immunity through adoptive transfer experiments, in which efferent gastric lymphocytes from immune animals responding to a challenge infection conferred partial protection to genetically identical naïve sheep (16). Experiments looking at the cellular profiles in the

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abomasal lymph node and abomasal tissue have shown changes in the quantity and proportions of both T and B cells at 3 and 5 days after infection with *T. circumcincta* (17), however the kinetics of the various cell types involved in the local cellular response has not been investigated to date.

In the present paper, we further characterize the immune response to a challenge infection of *Teladorsagia* by monitoring the phenotype and kinetics of the cells in the efferent gastric lymph of sheep which were immune or susceptible to infection, and by determining whether immune sheep reject the challenge infection within 48 h.

MATERIALS AND METHODS

Sheep

Three experiments were conducted using a total of 48 yearling sheep aged 10–12 months. All had been reared indoors under conditions designed to exclude accidental infection with nematode parasites.

Infective larvae

Infective L3 were from an anthelmintic susceptible *T. circumcincta* isolate which had been passaged through sheep at Moredun Research Institute for a number of years. Larvae were stored for up to 1 month at 4°C prior to administration. All infective larvae used within each experiment were derived from the same batch.

Surgical procedures, sample collection and post-mortem procedures

The common gastric lymph duct, which contains efferent lymph draining all four stomachs, was cannulated as detailed elsewhere (18). The sheep were fitted with an indwelling venous catheter placed in the jugular vein or the posterior vena cava. Collection, sampling and re-infusion of lymph, and post-mortem procedures were carried out as previously reported (11).

Worm counting and staging

Sub-samples (5%) of abomasal washings and mucosal digests were stained by the addition of a concentrated iodine solution, washed over a 38 µm sieve and searched under a stereomicroscope. The number of male, female and sexually undifferentiated EL4 was recorded.

Statistical methods

Arithmetic mean with standard errors are shown throughout. Parasite counts and percentage EL4 were compared by Student's *t*-test. Cell numbers were compared using Student's *t*-test, and, after log transformation, by repeated measure models (Genstat, VSN International Ltd, Hemel Hempstead, UK). $P < 0.05$ is regarded as the significant level of probability throughout.

Design of experiments

Three trials designated Experiments 2, 3 and 4 were conducted (Table 1), so numbered because they were part of a larger series of trials sharing the same general design (11).

All three experiments contained a group of previously infected sheep which had received a trickle immunizing infection of 2000 infective *Teladorsagia* larvae three times per week for 2 months. Experiments 2 and 4 also contained control sheep which did not receive the trickle infection. All sheep were challenged with a single dose of 50 000 *Teladorsagia* larvae 7 days after receiving fenbendazole to remove any remnants of the trickle infection from the previously infected animals. In experiments 2 and 3 the gastric lymph ducts of 10 sheep in each group were cannulated in the interval between anthelmintic treatment and challenge.

Experiment 2, which contained 30 sheep, has been described in detail before [Experiment 2 in (11)]. It compared the number and state of development of worms recovered from previously infected or control sheep killed 5, 10 or 21 days following challenge (Table 1) in relation to their lymph IgA responses. Here, we describe changes in the lymph cell traffic of three previously infected and four control sheep from that trial for 10 and 21 days after challenge, respectively. Previously infected sheep were killed 10 days after challenge as it is known from prior experiments using this model that the major manifestations of immunity to the parasite (worm loss and arrested development), as well as the cellular and humoral responses occur within 10 days (3,11).

Experiment 3 consisted of six previously infected sheep killed 10 days after challenge. Gastric lymph was successfully obtained from four of these and their cell traffic data was pooled with that of the three previously infected sheep in Experiment 2.

Experiment 4 contained six previously infected and six control sheep. Both groups were killed 2 days after challenge to determine whether worm loss had occurred by this time point. Lymph was not collected during this experiment.

Table 1 Design of experiments

Treatments (relative to day of challenge)											
Experiment	Breed	Group	Trickle infection ^a	Fenben-dazole (5 mg/kg)	Cannulate gastric lymph duct	Challenge infection ^b	Number killed				Main purpose
			-62 to -9	-7	-6 to -2	0	2	5	10	21	
2	SxD	Previously infected	+	+	+	+	–	6	6	–	To provide lymph cell traffic data from immune and naïve sheep post-challenge
		Control	–		+		–	6	6	6	
3	SxD	Previously infected	+		+		–	–	6	–	Does worm loss occur by day 2 post-challenge?
4	LxB	Previously infected	+		–		6	–	–	–	
		Control	–		–		6	–	–	–	

^a2000 *T. circumcincta* L3 three times per week for 8 weeks; ^b50 000 *T. circumcincta* L3. SxD = Suffolk tup, Dorset ewe; LxB = Leicester tup, Scottish Blackface ewe.

Immunofluorescent surface staining of lymphocytes

Lymph was diluted 1 : 500 in Coulter® Isoton® II Diluent (Beckman Coulter Inc., Fullerton, CA, USA) using a Coulter Diluter (Coulter Electronics Ltd, Luton, UK) and total (>3 µm diameter) and large (>9 µm diameter) lymphocyte numbers counted using a Z1 Coulter® Particle Counter (Beckman Coulter) Counter. 1×10^7 cells were centrifuged for 5 min at 1500 rpm, and the pellet washed by resuspending in 5 mL FACS buffer [PBS, 5% (v/v) foetal calf serum, 0.02% (w/v) NaN₃] and centrifuging again at 1500 rpm for 5 min. After a further wash in 5 mL FACS buffer the final pellet was resuspended in 1 mL FACS buffer. Live/dead discrimination using phase contrast was performed on early efferent lymph samples before staining and showed very little cell death, and examination of nigrosin stained lymphocytes also revealed negligible cell death. Fifty microlitres washed lymphocytes, at 1×10^7 cells/mL, were added to round-bottomed 96-well plates (Bibby Sterilin Ltd, Staffs, UK) and spun for 1 min at 2000 rpm. The supernatant was discarded, and after vortexing briefly to resuspend the pellet 50 µL per well of primary antibody diluted in FACS buffer was added. Monoclonal antibodies that recognize border disease virus as isotype controls [clones VPM21 (isotype IgG1, 1/500 dilution) and VPM22 isotype IgG2a, 1/500 (19)], ovine CD2 [clone 36F, isotype IgG2a, 1/1000 (20)], CD4 [clone 17D, IgG1, 1/1000 (21)], CD8 [clone 7C2, IgG2a, 1/1000 (22)], γδ-T cell receptor [clone 86D, IgG1, 1/1000 (23)], CD25 [clone ILA111, IgG2a, 1/2000 (24)], major histocompatibility complex class II [MHC-II; clone VPM46, IgG2a, 1/1000 (25)], CD21 [clone CC21, IgG1, 1/10 (26)] and IgA (MCA628; Serotec, Oxford, UK,

IgG1, 1/1000) were used. Plates were incubated for 20 min at 4°C, centrifuged for 1 min at 2000 rpm, the supernatant discarded, then after vortexing briefly wells were washed twice with 200 µL FACS buffer. After the second wash, 50 µL goat anti-mouse Ig phycoerythrin conjugate secondary antibody (Invitrogen Corporation, San Diego, CA, USA) diluted 1/400 in FACS buffer were added to each well. Plates were incubated in the dark for 15 min at room temperature, then 150 µL FACS buffer was added to each well before centrifuging for 1 min at 2000 rpm. Wells were then washed in 200 µL FACS buffer then 200 µL PBS before fixing in 1% (w/v) paraformaldehyde in PBS and analysed within 24 h.

Flow cytometry for visualization of stained cells

Fluorescent data were acquired on a FACScan flow cytometer equipped with a 488 nm argon-ion laser and analysed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). A minimum of 10 000 cells were acquired for each sample. Lymphocytes were initially gated according to forward scatter and side scatter to eliminate debris and dead cells, then percentage positive for each marker calculated. Large cells were defined by a visually determined cut off according to forward scatter. Example FACS plots from one previously infected sheep at days 1 and 3 post-challenge are shown in Figure 1. An initial Fsc vs. Ssc plot was gated for small lymphocytes, R1; blasting lymphocytes (lymphoblasts), R2; and total lymphocytes, R3 (R3 = R1 + R2) (Figure 1a and b). Negative control samples with no primary antibody (Figure 1c and d), and isotype controls (Figure 1e and f) were then plotted as Fsc vs. fluorescence, gated with R3.

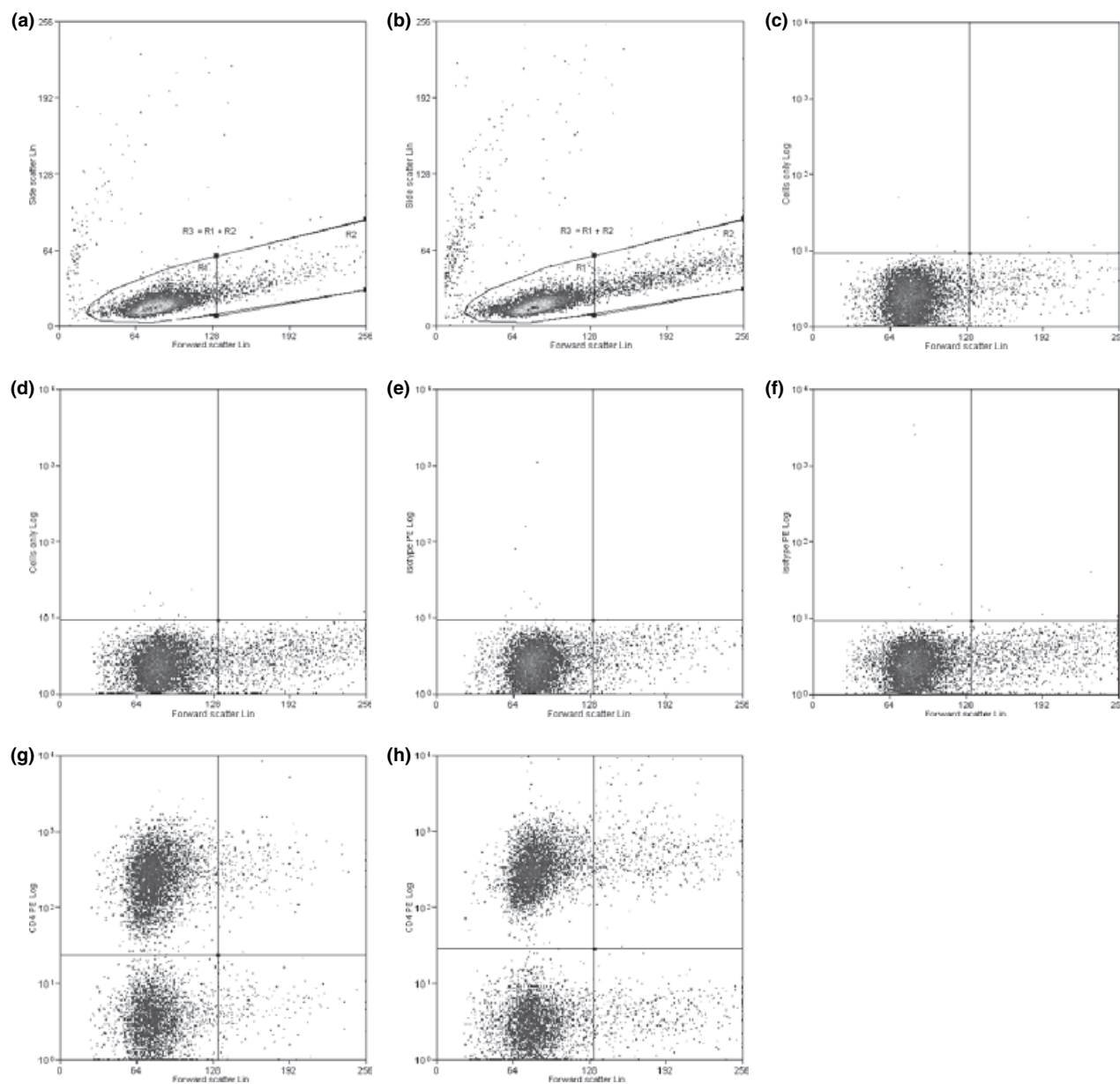


Figure 1 Example FACS plots from one previously infected sheep. a; c; e and g, 1 day post-challenge. b; d; f and h, 3 days post-challenge. a and b, Fsc vs. Ssc, no gate. R1 = small lymphocytes, R2 = lymphoblasts, R3 = total lymphocytes (R1 + R2). c and d, Fsc vs. fluorescence, cells only (negative control) sample. Gate = R3. e and f, Fsc vs. fluorescence, isotype control sample. Gate = R3. g and h, Fsc vs. fluorescence, CD4 stained sample. Gate = R3.

Quadrants were set with the Fsc cut-off for large cells as established during initial gating. Cells in the upper quadrants represent those positive for the staining antibody, and those in the upper right positive blast cells. The percentage of total cells positive for the isotype control antibodies was observed to be below 0.1% for almost all samples. Cells stained with anti-CD4, gated with R3, are shown in Figure 1g and h.

RESULTS

Parasitological data

In Experiments 2 and 4 significantly ($P < 0.01$) fewer worms were recovered from the previously infected sheep compared to their respective controls at each time point (Figure 2) and the number recovered from the previously

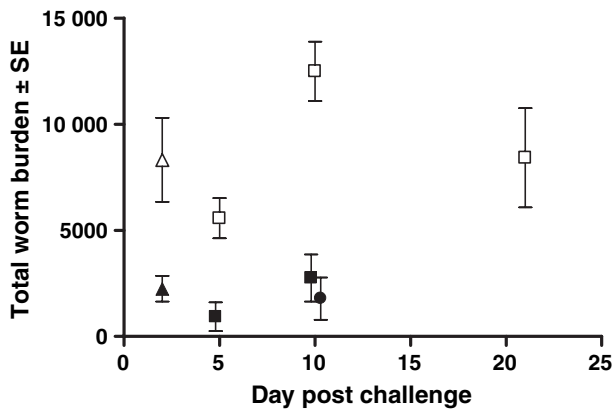


Figure 2 Worm burdens. Open symbols = controls. Closed symbols = previously infected. Squares = experiment 2. Circle = experiment 3. Triangles = experiment 4.

infected sheep in Experiment 3 was consistent with this data. This indicated that the previously infected sheep rejected the challenge larvae within 48 h.

Lymph flow

Lymph was collected until day 21 from 4 control sheep, and, except for 1 animal which stopped flowing on day 7, until day 9 or 10 from 7 previously infected sheep. Data from the sheep which stopped flowing at day 7 has been included in the group mean calculations for the relevant time points.

The overall mean lymph flow rate was 14.8 ± 0.8 mL/h. No significant changes with time or between groups were observed.

Lymph cell output

Total cells

In the control sheep the mean rate of total cell output increased from $1.55 \pm 0.31 \times 10^8$ cells/h on day 0 to $2.63 \pm 0.31 \times 10^8$ cells/h by day 3, however this was not a significant increase ($P = 0.08$) (Figure 3a). The mean rate of total cell output then remained elevated until day 10, thereafter falling to between 1.0 and 1.5×10^8 /h, similar to prechallenge values.

In previously infected sheep mean total cell output was $2.96 \pm 0.62 \times 10^8$ cells/h on day 3 compared to $1.86 \pm 0.63 \times 10^8$ cells/h on day 0, a significant increase ($P < 0.05$). No significant difference was found between the groups. Total cell output appeared to decrease slightly in both groups towards the end of the experiment.

Percentage blast cells

Prior to the challenge infection the proportion of lymphoblasts was similar in both groups with a mean of about 3% (Figure 3b). After challenge this value increased steadily in both groups, increasing to approximately 12% on day 8 in the previously infected, and peaking at approximately 14% on day 17 in the controls. No significant differences were observed between the groups except on day

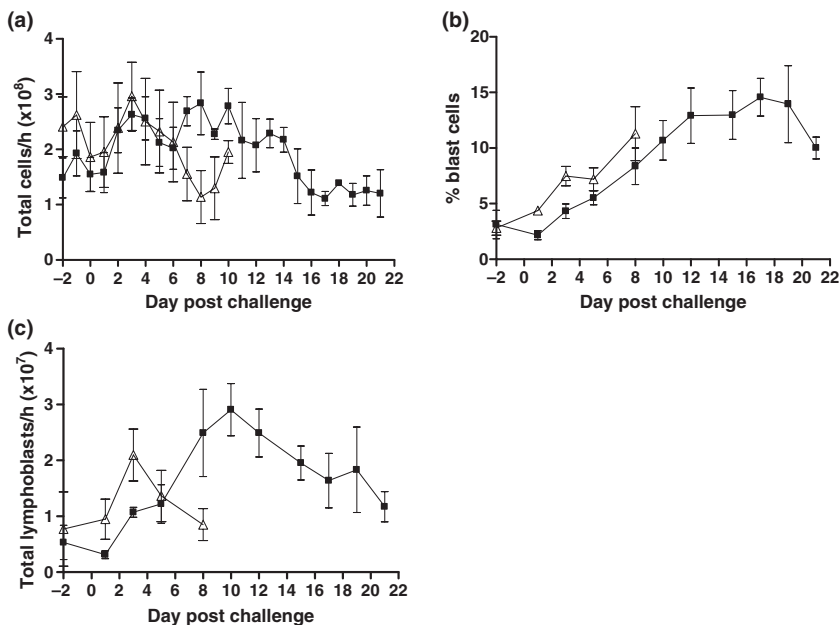


Figure 3 Cell output. Closed squares = controls. Open triangles = previously infected. (a) Total cell output per hour. (b) Percentage lymphoblasts. (c) Total lymphoblast output per hour.

1 when the percentage was significantly higher ($P < 0.05$) in the previously infected sheep.

Total lymphoblasts

In the previously infected sheep the rate of lymphoblast output increased significantly ($P = 0.05$) from $0.95 \pm 0.36 \times 10^7$ cells/h on day 1 to peak at $2.10 \pm 0.46 \times 10^7$ cells/h on day 3, then decreased almost to prechallenge levels by day 8 (Figure 3c). In the control sheep lymphoblast output rose more slowly after challenge, from $0.31 \pm 0.07 \times 10^7$ cells/h on day 1 to a peak of $2.91 \pm 0.47 \times 10^7$ cells/h ($P = 0.01$) on day 10, and then decreased throughout the remainder of the experiment (Figure 3c). As the most significant changes occurred within the blast cell population, and these are most likely to be the cells responding to the parasite infection, the cell-surface marker staining results are focused on lymphoblasts.

Proportion of T and B lymphocytes

In the previously infected group, the mean proportion of total lymphocytes expressing the cell surface markers CD4, CD8, γ/δ T-cell receptor and CD21 at 1 day post-challenge was 50%, 21%, 10% and 15% respectively, and in the control group was 54%, 17%, 9% and 30% (Table 2). Large between animal variation was observed, however the proportions of cell types in the efferent lymph did not differ between the two groups. Other than a slight decrease in CD4⁺ cells towards the end of the experiment in both groups, no changes over time in the proportion of cells expressing these markers was observed.

T cells

The response of lymphoblasts expressing the pan T-cell marker, CD2, was significantly different ($P = 0.032$) between the two groups (Figure 4a). CD2⁺ blasting cells in the previously infected group rose from

$0.43 \pm 0.15 \times 10^7$ cells/h on day 1 to peak on day 3 at $1.38 \pm 0.33 \times 10^7$ cells/h, while in the control group they initially increased more slowly, from $0.18 \pm 0.03 \times 10^7$ cells/h on day 1, peaking on day 10 at $2.36 \pm 0.60 \times 10^7$ cells/h. In each group the peak CD2⁺ lymphoblast output was significantly higher than at 1 day post-challenge ($P < 0.05$).

Similar between group differences were observed for CD4⁺ ($P = 0.049$, Figure 4b) and CD25⁺ ($P = 0.017$, Figure 4c) lymphoblast outputs which peaked in the previously infected group on day 3 at 1.02 ± 0.27 , and $0.97 \pm 0.24 \times 10^7$ cells/h respectively. In contrast, CD4⁺ and CD25⁺ lymphoblasts in the control group did not peak until day 10 with outputs of 1.22 ± 0.17 and $1.77 \pm 0.26 \times 10^7$ cells/h respectively. In each group of sheep peak outputs of both cell types were significantly ($P < 0.05$) greater than the corresponding day 1 values. In addition, the mean peak CD25⁺ output from the naïve group was significantly higher than from previously infected group ($P = 0.05$).

The CD8⁺ lymphoblast response was also significantly different between the two groups ($P = 0.017$), with positive blasting cells in the previously infected group again peaking 3 days post-challenge at $0.26 \pm 0.07 \times 10^7$ cells/h (Figure 4d). However, this was not significantly different from the day 1 output of $0.18 \pm 0.07 \times 10^7$ cells/h ($P = 0.08$), possibly due to wide individual variation. In the control group, a trend similar to that seen with CD4⁺ and CD25⁺ blasts was observed, with the CD8⁺ lymphoblast output peaking at day 10 at $0.97 \pm 0.39 \times 10^7$ cells/h. However, statistical analysis again showed this to be not significantly more than day 1 levels ($P = 0.1$), and also not significantly more than the peak output of the previously infected group ($P = 0.17$), possibly due to the high degree of individual variation observed and the relatively small group size.

The pattern of gamma-delta T-cell receptor positive lymphoblast response was significantly different between the two groups over the first 8 days of infection ($P = 0.001$, Figure 4e), with increased output in the previously infected group at days 1 and 3 compared to the control group. Analysis of the control group over the whole experiment showed that levels of γ/δ TCR⁺ blast cells did not change significantly with time.

B cells

Absolute numbers of blasting cells expressing B-cell markers are shown in Figure 5, with lymphoblasts expressing CD21 shown in Figure 5(a). In the previously infected group, CD21⁺ blast cell output rose slightly from $0.28 \pm 0.14 \times 10^7$ cells/h on day 1 to $0.49 \pm 0.19 \times 10^7$ cells/h on day 5, then dropped to $0.18 \pm 0.09 \times 10^7$ cells/h

Table 2 Distribution of lymphocyte subsets at one day post-challenge in the efferent gastric lymph of naïve and previously infected sheep

Cell type	Naïve Mean % (range)	Previously infected Mean % (range)
T cells (CD2 ⁺)	71 (59–77)	71 (60–77)
CD4 ⁺	54 (48–62)	50 (41–60)
CD8 ⁺	17 (11–23)	21 (15–29)
γ/δ	9 (7–13)	10 (6–16)
B cells (CD21 ⁺)	30 (7–50)	15 (10–21)

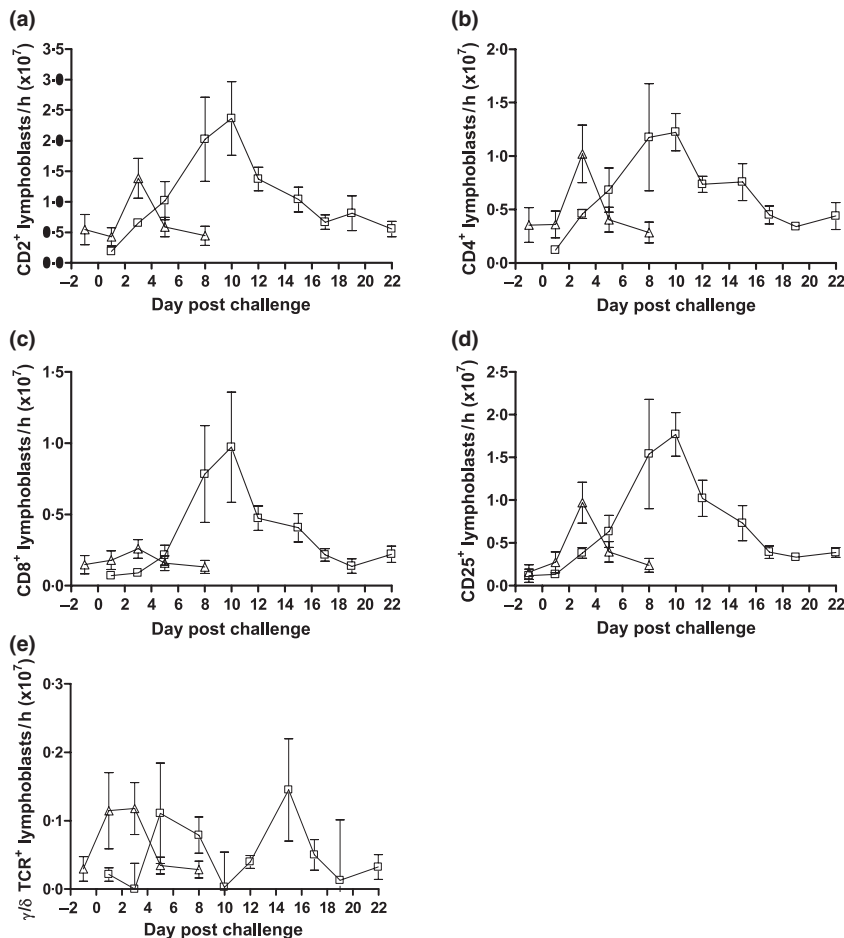


Figure 4 T-cell lymphoblast output per hour. Squares = controls. Triangles = previously infected. (a) CD2⁺. (b) CD4⁺. (c) CD8⁺. (d) CD25⁺. (e) γ/δ TCR⁺.

on day 8, but these changes were not significant. In the control group, CD21⁺ blast cell output rose significantly ($P = 0.01$) from $0.13 \pm 0.04 \times 10^7$ cells/h on day 1 to $1.04 \pm 0.17 \times 10^7$ cells/h on day 10, before decreasing back to prechallenge levels by day 19.

In the previously infected group, IgA⁺ blast cell output peaked at day 5 at $0.41 \pm 0.14 \times 10^7$ cells/h, significantly higher ($P = 0.04$) than day 1 ($0.09 \pm 0.03 \times 10^7$ cells/h), then dropped back to $0.12 \pm 0.06 \times 10^7$ cells/h by day 8 (Figure 5b). In the control group the IgA⁺ blast cell response appeared to rise slowly over time, from $0.08 \pm 0.04 \times 10^7$ cells/h on day 1 to $0.29 \pm 0.07 \times 10^7$ cells/h on day 22, the only point at which the output was significantly higher than day 1 ($P = 0.04$).

Figure 5(c and d) show the kinetics of the IgA⁺ blast cell response in relation to total lymph IgA concentrations which have been reported previously (11). In control sheep mean total IgA concentrations did not change significantly over the course of the experiment reflecting the general lack of an IgA⁺ blast cell response. In contrast a clear anamnestic total IgA response was observed in the previ-

ously infected sheep, which tracked the secondary IgA⁺ blast cell response.

MHC II positive cells

The response of lymphoblasts expressing MHC II on the cell surface is shown in Figure 5(e). Prechallenge this was in the range $0.3\text{--}0.6 \times 10^7$ cells/h, and was not different between the two groups. After challenge MHC II⁺ lymphoblast output increased in both groups, peaking at day 3 in the previously infected sheep at $1.26 \pm 0.29 \times 10^7$ cells/h, and at day 10 in the naïve animals at $2.06 \pm 0.43 \times 10^7$ cells/h before subsiding again.

DISCUSSION

The parasitology data from the three trials described in the present paper confirmed that, with this particular infection model, the previously infected sheep rejected most of the challenge dose which established in the control animals (11) and showed that this phenomenon had occurred within 2 days of infection. Worm loss, through

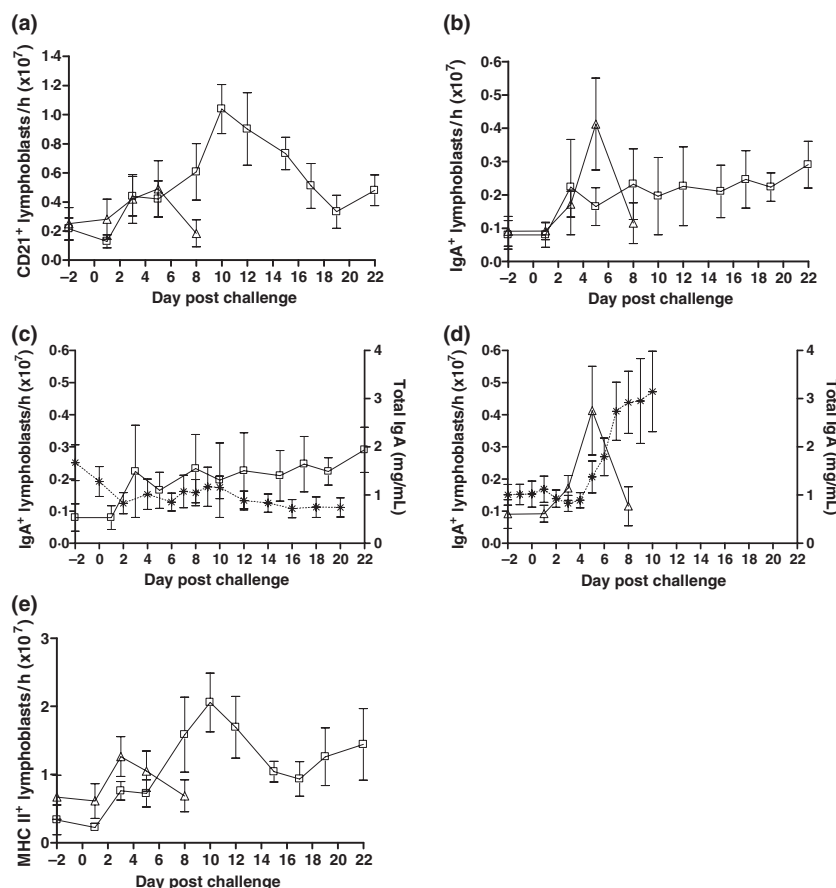


Figure 5 B-cell lymphoblast output per hour. Squares = controls. Triangles = previously infected. Solid lines = cells. Asterisks and broken lines = antibody. (a) CD21⁺. (b) IgA⁺. (c) Control IgA⁺ blast cells and total IgA. (d) Previously infected IgA⁺ blast cells and total IgA. (e) MHC 11⁺.

exclusion or expulsion of incoming larvae from the gastric pit, is the most important manifestation of immunity to *T. circumcincta*, and prior experiments suggested that it was the result of an active immune response rather than a passive state (10). In each group, more worms were recovered at 10 days post-infection than on day 5, which probably reflects the relative inefficiency of recovering worms from the gastric mucosa, as experienced previously (16).

In this paper, we followed the kinetics of the local cellular response in the efferent gastric lymph to infection with *T. circumcincta*. An increase in total lymphocyte output was observed in both groups after infection, corresponding to the 'recruitment phase' of the local inflammatory response (27). An increase in the percentage of large, blast cells in the gastric lymph, that is, cells responding to the parasitic infection, was also observed in both groups, however this occurred more rapidly in previously infected than in naïve animals. When this data was combined with the total cell output to calculate absolute blast cell traffic, the previously infected animals showed a rapid increase in large cell output, which peaked 3 days after challenge. A

significant increase in blast cell output was also observed in the animals undergoing primary infection, however it occurred much more slowly, not peaking until day 10. This agrees with previous findings where the local cellular response of previously infected sheep was observed to peak sooner than that of sheep undergoing a primary infection (2,3).

As expected, the cells in the efferent gastric lymph were almost exclusively lymphocytes; no cells positive for monocyte or macrophage specific markers were observed (data not shown). The proportion of T and B cells in efferent gastric lymph was comparable to that reported in lymph draining other lymph nodes (28,29), including some draining other mucosal surfaces (27,28,30,31). A slight drop in the percentage of CD4⁺ cells was observed in both groups towards the end of the experiment (data not shown), however other than this the cellular response manifested as an increase in total output of cells, and in particular blast cells, rather than as a change in the proportion of individual cell types.

Phenotypic analysis of the cells involved in the immune response, peaking at day 3 in previously infected sheep

and day 10 in naïve sheep, showed that they were predominantly T cells, and within this subset mainly CD4⁺. Depletion experiments have shown CD4⁺ T cells to be essential for immunity of sheep to *Haemonchus contortus* (32–34), and while similar experiments have not, to our knowledge, been carried out with *T. circumcincta*, our results suggest that CD4⁺ cells will also play an important role in immunity to this parasite.

CD4⁺ cell outputs were greater than CD8⁺ during the blast cell response of the previously infected sheep but peak CD8⁺ blast cell output was slightly higher in the primary infection, suggesting a predominant role for CD4⁺ cells in the memory response. CD25⁺ lymphoblast numbers corresponded to T lymphoblast numbers, which could reflect either T cells expressing CD25 (IL-2R) upon activation, or regulatory T cells, however labelling of multiple cell surface markers would be required to determine which.

The B-cell response occurred slightly later than the T-cell response, peaking 5 days after challenge in the previously infected animals. The peak IgA⁺ blast cell response almost matched the total B-cell blasts in this group, most likely because isotype switching to IgA had occurred during the trickle infection period. The same sheep also demonstrated a secondary lymph IgA response (11), which tracked these IgA⁺ blast cells approximately 2 days later. By 8 days post-challenge, IgA⁺ blast cell output had returned to prechallenge levels even although the amount of IgA in the lymph remained elevated. Presumably the source of the lymph IgA was plasma cells in the mucosa derived to at least some extent from the lymph IgA⁺ blast cells which had re-circulated to the gastric mucosa. CD21⁺ blast cells also subsided to prechallenge levels by day 8. By analogy with human plasma cells, which rarely express CD21 (CR2) (29,35), this change in phenotype was probably due to the loss of CD21 from terminally differentiated antibody secreting cells although this has not yet been confirmed for the sheep.

In the naïve group, the B-cell blast response was slower, not peaking until day 10, and a corresponding peak in IgA⁺ blast cells was not observed, as these animals were undergoing a primary immune response.

In this paper, we have characterized the phenotype and extent of the cell traffic response in the gastric lymph of immune or susceptible sheep after infection with *T. circumcincta*. These responses do not seem to have been monitored in this way before in lymph draining a mucosal surface. Pernthaner *et al.* (30) monitored cells in afferent and efferent intestinal lymph from sheep infected with *T. colubriformis* but did not document any changes other than a decrease in the proportion of CD4⁺ cells from 1 week after infection. Yen *et al.* (31) collected efferent

lymph draining the nasopharynx of sheep, and reported an increase in lymphocyte proliferation *ex vivo* following intranasal vaccination, but did not report any change in cell traffic.

In the present study, a memory response was observed in the immune animals consisting initially of blasting CD4⁺ cells, closely followed by IgA⁺ B cells and a corresponding increase in parasite specific lymph IgA. However, the worm loss preceded all these responses, indicating that none was directly responsible and that the immune effector mechanism was probably operating in the abomasal mucosa. The function of the lymph cell response may be to disseminate primed or memory cells to other mucosal surfaces including the abomasum, a contention supported by earlier adoptive transfer experiments (16), in which cells from immune sheep undergoing a response to infection conferred partial immunity to genetically identical, naïve sheep.

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4 Kinetics of the local immune response in the gastric lymph of lambs after primary and challenge infection with *Teladorsagia circumcincta*.

Aileen M Halliday, Heather C McAllister and W David Smith (2010). *Parasite Immunol*, **32**: 81-90.

This is the final paper in a series of 3 papers revisiting the model of efferent gastric lymph duct cannulation in order to gain a greater understanding of the timing and nature of the ovine immune response to infection with *Teladorsagia circumcincta*.

Previous studies using this model in the 1980s revealed that previously infected lambs undergoing a challenge infection with *T. circumcincta* were less able to mount an effective immune response when compared to adult sheep undergoing the same regimen (Smith *et al* 1985). Research carried out using other gastro-intestinal nematodes of sheep, such as *Haemonchus contortus* and *Trichostrongylus colubriformis* has also identified reduced capacity of lambs to mount an effective immune response to these parasites, when compared to adult sheep (Colditz *et al* 1996, Gregg *et al* 1978, Smith and Angus 1980).

The experiments in this paper were designed to assess the timing and components of the immune response of primary and challenge infected lambs infected with *Teladorsagia circumcincta*, replicating the experimental infection model used with adult sheep as described in the first 2 papers in the series, in order to compare the nature and effectiveness of both the humoral and cellular response between the 2 ages of sheep.

Contribution to the work

The candidate was involved in all areas of experimental design and planning, surgery to cannulate the gastric lymph ducts of lambs, daily lymph and blood collection, parasitological analyses, and carried out humoral and cellular assays and data processing and analysis. Results were presented at local, national and international meetings.

Kinetics of the local immune response in the gastric lymph of lambs after primary and challenge infection with *Teladorsagia circumcincta*

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SUMMARY

Groups of 5-month-old lambs which had been trickle infected with *Teladorsagia circumcincta* for 8 weeks then drenched, and worm-free control lambs were challenged with 50 000 *T. circumcincta* L3s. From 10 days later fewer parasites were recovered from the previously infected sheep, and secondary cellular and humoral responses were observed in the gastric lymph. Increases in CD4⁺ and CD25⁺ T lymphoblast traffic on day 3, followed by CD21⁺ and IgA⁺ lymphoblasts on day 5, and an increase in total and parasite specific IgA concentrations peaking on day 6 were observed in previously infected lambs. Similar peaks in lymphoblast output were not observed until days 10–12 in the control lambs. This data was highly comparable with that obtained recently from yearling sheep subjected to an identical infection-challenge regime, and contrasted with that obtained from similar experiments in the 1980s when 4^{1/2}-month-old previously infected lambs were more susceptible to and had much weaker immune responses to challenge than 10-month-old sheep. The fact that 40% fewer larvae were given during the trickle infection regime in the four recent trials is offered as an explanation for this difference.

Keywords acquired immunity, gastric lymph, lambs, *Teladorsagia circumcincta*

INTRODUCTION

Teladorsagia circumcincta is an abomasal nematode parasite of sheep, and is a serious problem in temperate areas both in terms of animal welfare and economic loss.

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Current control methods rely on the use of anthelmintic drugs; however, resistance to these drugs is wide-spread and increasing, and isolates of *T. circumcincta* have been identified which display phenotypic resistance to several classes of anthelmintic (1–3).

Sheep which have been exposed to *Teladorsagia* can acquire protective immunity, so vaccination is viewed as a possible alternative method of control.

Both cellular and humoral responses have been associated with protective immunity. Previously infected adult sheep undergo a local blast cell response in the first few days after challenge infection, and these cells adoptively transferred partial immunity to genetically identical parasite naïve recipients (4–6). Immediate type hypersensitivity responses have also been associated with immunity, and correlated with a reduction in worm burden (7–9), whereas the mucosal IgA response has been associated with stunted growth and reduced fecundity of developing worms (10–13).

We have developed an experimental infection model in which previously infected yearling sheep acquired a substantial degree of protective immunity to *T. circumcincta* compared to naïve animals undergoing a primary infection (5,10,14,15). In this paper, we have repeated these experiments in 5-month-old lambs, to compare the responses of the two age groups. This investigation was motivated by the fact that age-related immunity to gastrointestinal nematode parasites has been widely documented in sheep, yet the underlying reasons are poorly understood. Thus, compared to adult sheep, lambs develop impaired immunity to natural nematode infections or following immunisation with irradiated larvae (16–22), despite being capable of mounting protective immune responses to a variety of vaccines including ones containing nematode intestinal antigens (23). More specifically, prior experiments with a very similar *Teladorsagia*/gastric lymph model showed that young lambs were more susceptible than yearlings to infection and mounted measurably lower secondary immune responses (5,11).

MATERIALS AND METHODS

Sheep

Two experiments were carried out involving a total of 66 lambs aged 5 months at time of challenge. All had been reared indoors under conditions designed to exclude accidental infection with nematode parasites.

Infective larvae

Infective larvae were from an anthelmintic susceptible *T. circumcincta* isolate which had been passaged through sheep at Moredun Research Institute for a number of years. Larvae were stored for up to 1 month at 4°C prior to administration. All infective larvae used within each experiment were derived from the same batch.

Surgical procedures, sample collection and post-mortem procedures

The common gastric lymph duct, which contains efferent lymph draining all four stomachs, was cannulated as detailed elsewhere (24). The sheep were fitted with an indwelling venous catheter placed in the posterior vena cava. Collection, sampling and re-infusion of lymph, and post-mortem procedures were carried out as previously reported (10).

Worm counting, measuring and staging

Worm counts were carried out as detailed elsewhere (10). A random sample of approximately 50 parasites obtained from each animal killed on day 10 of Experiment 6 was measured by a Camera Lucida under 10× magnification.

Sexually undifferentiated worms measuring <1.5 mm were classified as EL4, longer parasites were designated developing worms.

Statistical methods

Arithmetic means with standard errors are shown throughout. Parasite counts and percentage EL4 were compared by Student's *t*-test. Frequency distributions of male and female worm lengths were made for individual sheep and group means were calculated from these. Immunoglobulin concentrations and cell numbers were compared using Student's *t*-test, and, after log transformation, by repeated measures (Genstat). Correlations between worm burdens and antibody titres were carried out using Spearman's rank correlations. *P* < 0.05 was regarded as the significant level of probability throughout.

Design of experiments

Two trials designated Experiments 5 and 6 were conducted (Table 1), so numbered as they were part of a larger series of trials sharing the same design. Both experiments contained a group of sheep which had received a trickle immunising infection of 2000 *T. circumcincta* infective larvae three times per week for 8 weeks, and a group of control sheep which had not received the trickle infection. All were dosed with fenbendazole one week prior to challenge with a single dose of 50 000 infective larvae, with surgery to cannulate the gastric lymph duct being carried out on 10 sheep in each experiment during the intervening week. Sheep were killed on days 5, 10 or 21 post-challenge. It was known from prior work using this experimental model that in previously infected sheep the cellular and humoral immune responses in lymph all occurred by

Table 1 Design of experiments

			Treatments (relative to day of challenge)							
Expt	Breed	Group	Trickle infection (-62 to -9) ^a	Fenbendazole (5 mg/kg) (-7)	Cannulate gastric lymph duct (-6 to -2)	Challenge infection (0) ^b	Number killed			Main purpose
							(5)	(10)	(21)	
5	L × B	Previously infected	+	+	+	+	6	5	5	To provide data on parasitology and lymph parameters from immune and naïve lambs post-challenge
		Control	-		+		6	5	4	
6	S × D	Previously infected	+		+		6	6	6	
		Control	-		+		6	5	6	

^a2000 *Teladorsagia circumcincta* L3 three times per week for 8 weeks; ^b500 000 *T. circumcincta* L3.

L × B = Leicester tup, Scottish Blackface ewe; S × D = Suffolk tup, Dorset ewe.

day 9 after challenge. Therefore, lymph collection from the previously infected lambs was stopped after 10 days.

Determination of blast cells

Large cells or lymphoblasts were determined as those with a diameter of $>9\ \mu\text{m}$ when measured by Coulter Counter, with small lymphocytes represented as those with a diameter of between 3 and $9\ \mu\text{m}$. During FACS analysis, small cells were those appearing within region R1 on a control sample Fsc vs. Ssc plot (Figure 1), blast cells were designated as the gated lymphocytes which fell within region R2 and total lymphocytes within R3 ($=\text{R1} + \text{R2}$). Downstream FACS analyses of stained cells were gated to contain only those cells present in R3.

Immunofluorescent surface staining of lymphocytes and flow cytometry for visualisation of stained cells

Surface staining of lymphocytes from gastric lymph, and flow cytometry, were carried out as detailed previously (6). Monoclonal antibodies that recognise border disease virus as isotype controls (clones VPM21 (isotype IgG1, 1/500 dilution) and VPM22 (isotype IgG2, 1/500) (25)), ovine CD4 (clone 17D, IgG1, 1/1000 (26)), CD8 (clone 7C2, IgG2a, 1/1000 (27)), $\gamma\delta$ T cell receptor (clone 86D, IgG1, 1/1000 (28)), CD25 (an activated T cell marker, clone ILA111, IgG2a, 1/2000 (29)), CD21 (a pan B cell marker, clone CC21, IgG1, 1/10 (30)) and IgA (MCA628, Serotec, Oxford, UK, IgG1, 1/1000) were used. The percentage of total cells positive for the isotype control antibodies was observed to be below 0.15% for 99.3% of all samples.

Estimation of antibody titres

Detection and quantification of antibody in the gastric lymph was carried out as detailed previously (10). Briefly,

total IgA was measured using a sandwich ELISA, with purified sIgA as a standard. Antigen specific IgA was measured for both somatic L4 antigen, and L4 excretory/secretory (ES) products, with a positive reference sample included on each plate.

RESULTS

Parasitology

Previously infected lambs had significantly ($P < 0.05$) fewer parasites than controls on day 10 after challenge in both experiments (Figure 2a). However, on day 5 a significant difference ($P < 0.05$) was only observed within Experiment 5. No significant differences in worm burdens between previously infected and control lambs were observed on day 21.

There appeared to be higher worm counts at day 10 than day 5 in both of the current experiments, which may reflect the relative inefficiency of recovering day 5 larvae from the gastric mucosa, as observed previously (4). It would also appear that the overall 'take' of the worms in Experiment 5 was lower than in Experiment 6 (day 10 worm counts: Expt 5, 9080; Expt 6, 15 332).

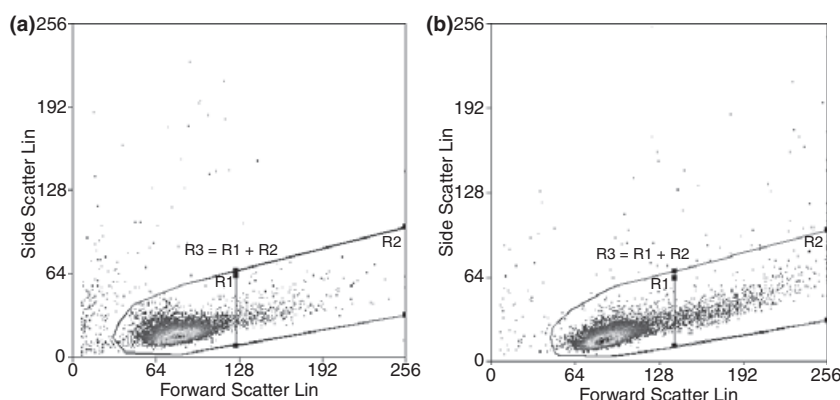
In both experiments, the percentage of arrested early L4s recovered at day 10 was higher in previously infected lambs than in controls (Figure 2b), but this difference was not statistically significant due to the large degree of individual variation.

In Experiment 6 significantly ($P < 0.005$) shorter developing male and female worms were recovered from previously infected compared to control lambs on day 10 (Figure 2c).

Duration of lymph flow and pooling of lymph data

Due to the small group sizes and the finding that the parasitology outcome was very similar within Experiments

Figure 1 Example FACS plot of control sample from one naïve sheep. (a) 1 day post-challenge. (b) 10 days post-challenge. R1 = small lymphocytes. R2 = lymphoblasts. R3 = total lymphocytes (R1 + R2).



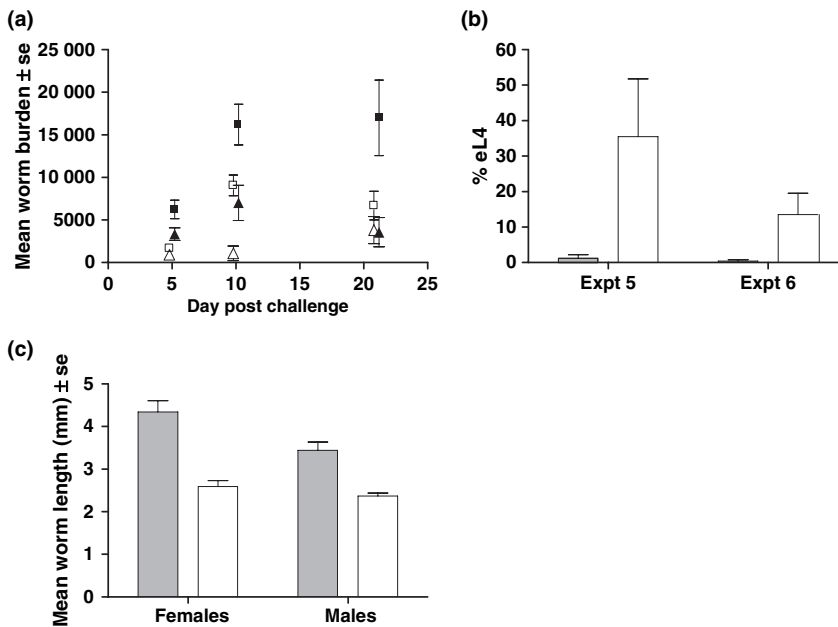


Figure 2 Parasitology data from lambs. (a) Worm burdens; squares = controls, triangles = previously infected; open symbols = experiment 5, closed symbols = experiment 6. (b) % EL4 recovered at day 10; grey bars = controls, white bars = previously infected. (c) Mean group length of developing worms recovered at day 10; grey bars = controls, white bars = previously infected.

5 and 6, lymph data of previously infected and control sheep were pooled, regardless of experiment.

Lymph flow was maintained in five previously infected and eight control animals until day 10. Three controls produced lymph until day 21 but flow ceased between days 10 and 14 in the remaining 5. All data was included in the group means for the available time points.

Lymph flow rates

At the time of challenge, the group mean lymph flow rates of control and previously infected lambs were 11.3 ± 2.7 and 8.0 ± 2.4 mL/h respectively, ($P > 0.05$). There was a trend towards increased lymph flow in both groups after challenge; however, this was only significant ($P < 0.01$) in the control group from day 6, when it reached 18.8 ± 3.5 mL/h.

Cell output in gastric lymph

Prior to challenge the group mean total cell output for both previously infected and control lambs was in the range of 1.6 – 2.2×10^8 cells/h (Figure 3a). This increased significantly ($P < 0.05$) after challenge in the previously infected group, peaking at $3.06 \pm 0.5 \times 10^8$ cells/h on day 3 before returning to pre-challenge levels. In the control group, the total cell output was slower to increase, peaking on day 6 at $2.72 \pm 0.4 \times 10^8$ cells/h ($P = 0.01$), but the increase was more sustained and did not decline to pre-challenge levels until day 10. The percentage of large or blasting cells in the lymph was measured by Coulter

counter (Figure 3b) and FACS (Figure 3c). Both methods showed that both treatment groups responded with an increase in the proportion of blast cells following challenge, but this occurred faster in the previously infected group, peaking at days 3–5 following challenge, whereas not becoming apparent until days 6–8 in the control group. Total cell output and the percentage lymphoblasts measured by FACS were combined to give the absolute lymphoblast output per hour (Figure 3d). In the previously infected group this peaked around days 3–5 at $\sim 2.2 \times 10^8$ cells/h, although this was not significantly higher than pre-challenge values due to two individuals having a high proportion of lymphoblasts pre-challenge. In the control group absolute lymphoblast output peaked at day 10 with $3.25 \pm 0.8 \times 10^8$ cells/h, significantly higher than the pre-challenge output of around 0.5×10^8 cells/h. In both groups, the lymphoblast output had returned to pre-challenge levels by the end of the experiment.

Phenotypic analysis of gastric lymph blast cells

T cells

A CD4⁺ blast cell response was observed in both the control and previously infected groups of lambs, with a repeated measures model showing strong evidence of a difference in the pattern of responses over time between the two groups ($P < 0.001$). In the control group, the CD4⁺ blast cell response peaked at day 10 at $1.58 \pm 0.19 \times 10^7$ cells/h (Figure 4a), and in the previously infected group peaked at day 3 at $0.9 \pm 0.24 \times 10^7$ cells/h (Figure 4b).

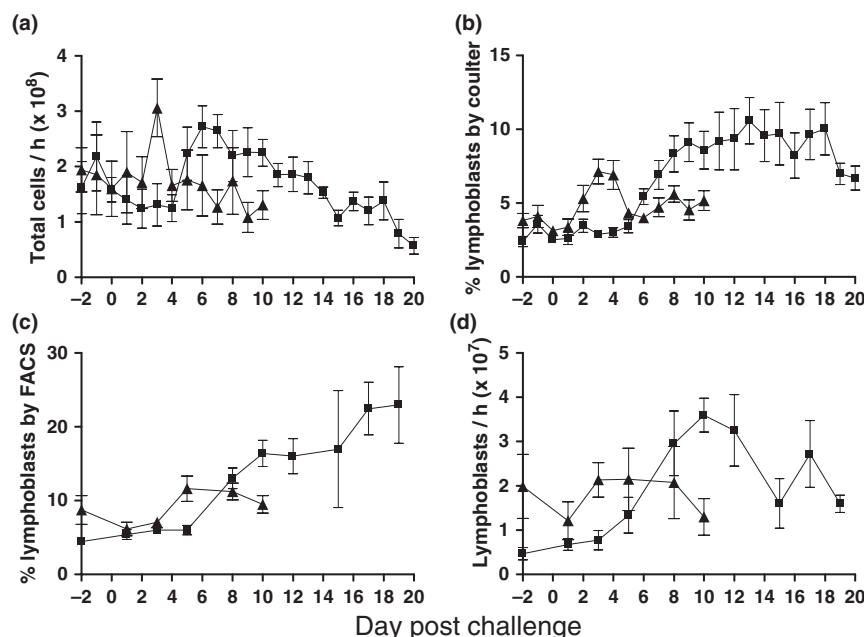


Figure 3 Cell output in gastric lymph of lambs. Squares = controls. Triangles = previously infected. (a) Total cell output per hour. (b) Percentage lymphoblasts measured by Coulter counter. (c) Percentage lymphoblasts measured by FACS. (d) Total lymphoblast output per hour.

A CD8⁺ blast cell response was observed in the controls but not in the previously infected group (Figure 4c, d).

No significant changes were observed in the gamma-delta T cell receptor positive blast cell response of either group of lambs (Figure 4e, f), the increase in mean output observed on day 12 in the controls being caused by a single outlier animal. Prior to challenge, three of the previously infected lambs had elevated levels of γ/δ TCR⁺ blast cells (Figure 4f), however these had subsided by day 1.

The CD25⁺ blast cell response was similar to CD4, with strong evidence of a difference in pattern of response between the two groups ($P < 0.001$). Naïve lambs showed an increase in CD25⁺ blast cells from day 5, peaking at day 10 at $1.76 \pm 0.3 \times 10^7$ cells/h (Figure 4g). In the previously infected group the response occurred sooner, peaking on day 3 at $1.30 \pm 0.3 \times 10^7$ cells/h (Figure 4h).

B cells

In the naïve group a CD21⁺ blast cell response was observed which peaked on day 10 at $0.76 \pm 0.1 \times 10^7$ cells/h (Figure 5a), significantly ($P < 0.05$) higher than the pre-challenge output of $0.16 \pm 0.1 \times 10^7$ cells/h. The same response occurred more quickly in the previously infected lambs peaking on day 5 at $0.73 \pm 0.2 \times 10^7$ cells/h (Figure 5b). The repeated measures model showed inconclusive evidence ($P = 0.068$) of a difference in the pattern of responses between the two groups, due in part to relatively high estimated standard errors.

IgA⁺ blast cell output was increased 10 and 12 days after the naïve lambs were infected, peaking at $0.51 \pm 0.1 \times 10^7$ cells/h (Figure 5c), and in the previously infected group peaked on day 3 at $0.23 \pm 0.1 \times 10^7$ cells/h (Figure 5d). This led to strong evidence of a difference in pattern of response over time between the two groups ($P < 0.001$).

IgA content of gastric lymph

Total IgA

Before challenge mean total IgA concentrations in the efferent gastric lymph of control and previously infected lambs were similar, at 0.53 ± 0.2 and 0.34 ± 0.04 mg/mL respectively (Figure 6a, b). In the control group this did not change significantly over the course of the experiment, whereas in the previously infected group a secondary IgA response was observed, rising on day 5 to a peak of 2.87 ± 1.3 mg/mL on day 6, before declining to almost pre-challenge levels by day 10.

Parasite specific IgA

IgA antibodies specific for *T. circumcincta* L4 antigen followed the pattern of response observed for total IgA (Figure 6c, d). Concentrations in both naïve and previously infected lambs were close to background values prior to challenge, but by day 3 a secondary response was evident in the previously infected group, peaking at day 6. The control group did show a slight increase in parasite

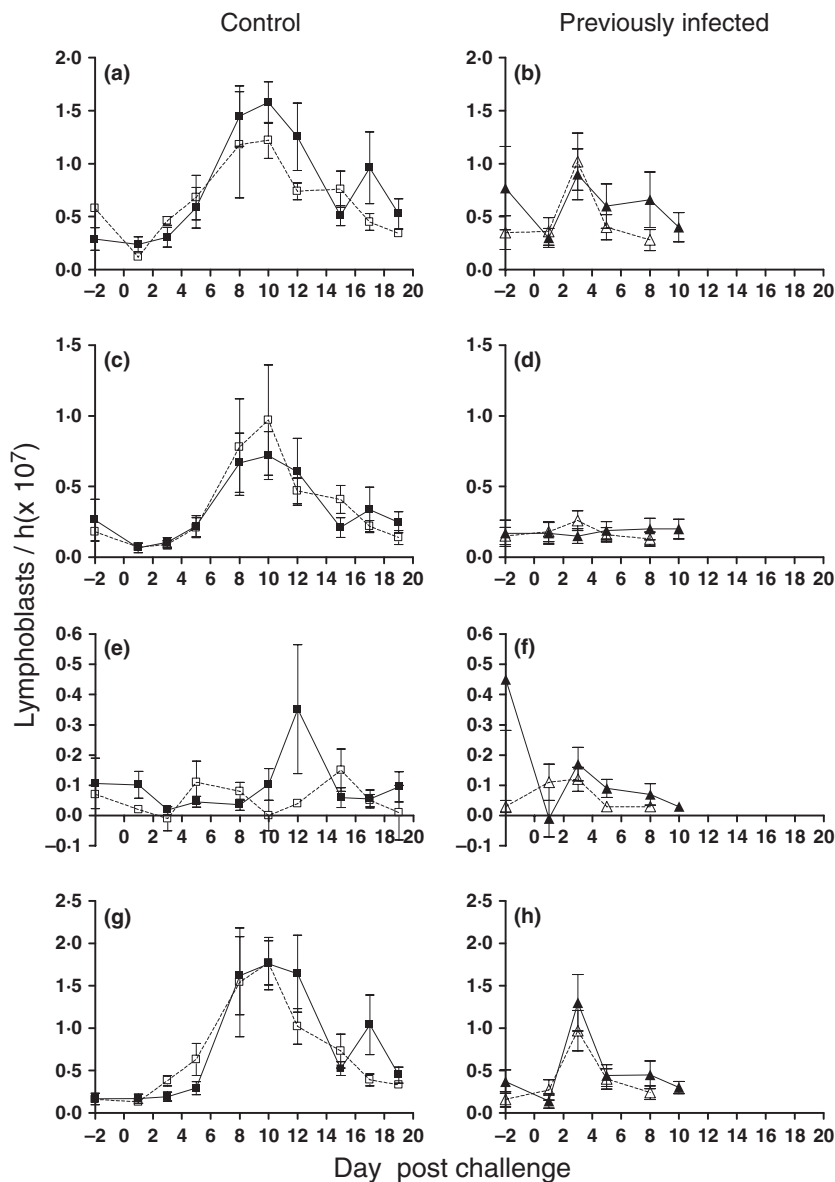


Figure 4 T cell lymphoblast output per hour. Squares = controls. Triangles = previously infected. Closed symbols with solid lines = lambs (current data). Open symbols with dotted lines = equivalent data from yearling sheep to allow direct comparison (6). (a) and (b) CD4⁺ blast cells. (c) and (d) CD8⁺ blast cells. (e) and (f) γ/δ TCR⁺ blast cells. (g) and (h) CD25⁺ blast cells.

specific IgA towards the end of the experiment but this was not significantly above pre-challenge levels.

DISCUSSION

The two experiments described in this paper examined the parasitology and local immune responses of lambs following infection with *T. circumcincta* within the context of an established experimental infection model. This discussion will first focus on the results that were obtained, and then compare these to data from yearling sheep undergoing an identical regime in two earlier trials within this series of experiments (6,10). Finally, all of those results will be

examined in the context of similar age comparison experiments which were carried out in the 1980s (11).

The previously infected lambs in the current experiments were partially immune to the challenge infection which established in the controls. They had significantly lower worm burdens from 10 days after challenge; more arrested early L4s and shorter developing worms.

Analysis of the immunological responses showed an increase in total cell output and percentage blast cells in the gastric lymph of both groups of lambs after infection; however, this occurred faster in the previously infected group than in the controls. Absolute blast cell output per hour in the gastric lymph mirrored this, increasing sooner

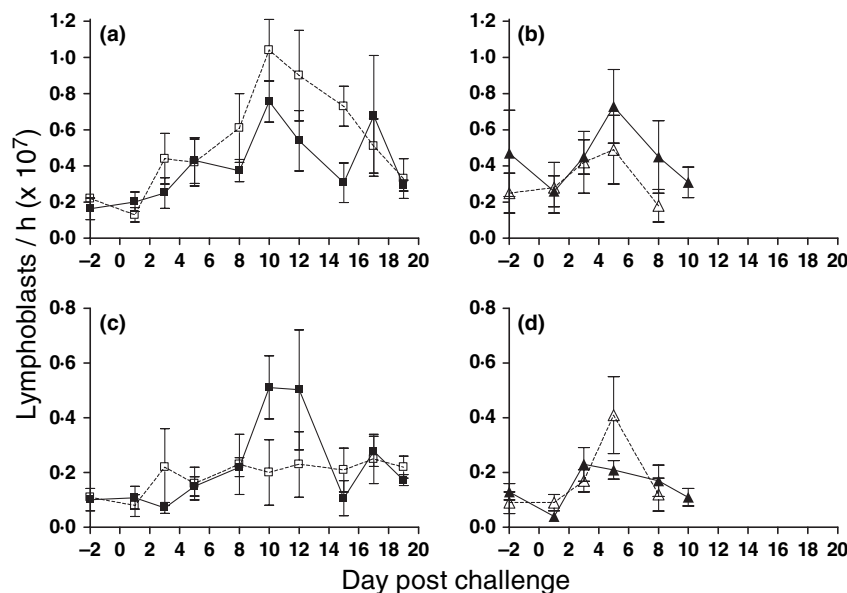


Figure 5 B cell lymphoblast output per hour. Squares = controls. Triangles = previously infected. Closed symbols/solid lines = lambs. Open symbols/dotted lines = yearlings (6). (a) and (b) CD21⁺ blast cells. (c) and (d) IgA⁺ blast cells.

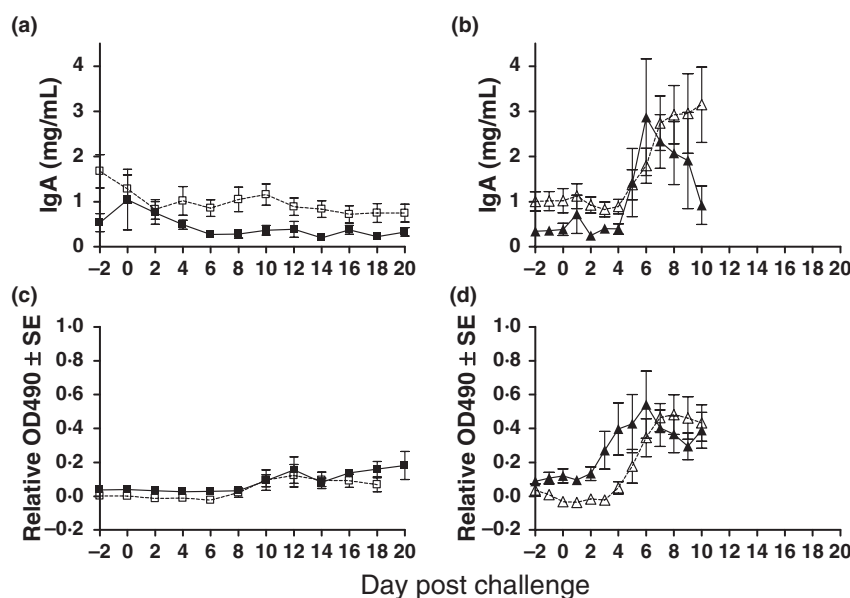


Figure 6 Total and parasite specific IgA. (a) and (b) Total IgA. (c) and (d) L4 somatic extract specific IgA. Closed symbols/solid lines = lambs. Open symbols/dotted lines = yearlings (6).

after challenge and peaking at day 3 in the previously infected group, compared to day 10 in the controls.

Phenotypic analysis of the blast cell response showed that it consisted of both T and B lymphocytes. The T cell response peaked 3 days after challenge in the previously infected group, and consisted predominantly of CD4⁺ cells. In the control group, the T cell response did not peak until 10 days after challenge, and was composed of both CD4⁺ and CD8⁺ T cells. The B cell and IgA⁺ blast cell response was also observed to occur sooner in the previously infected animals, again peaking at 3 days after

challenge, with the control group not peaking until day 10. Soluble IgA detected in the gastric lymph of previously infected lambs tracked the increase in IgA⁺ blast cells, rising after 3–5 days, and peaking on day 6. No significant increase in IgA was observed in the gastric lymph of controls.

The results from these lamb experiments were compared to previously published data obtained from yearling sheep which had undergone the same infection regime as part of the same series of studies (6,10). The degree of immunity the lambs demonstrated to the challenge infection was

indistinguishable from that shown in the yearling trials. Comparison of the immunological parameters also showed a high degree of similarity between lambs and adults, both in terms of the magnitude and timing of the blast cell response, and the phenotypes of the blasting cells (to simplify this comparison yearling and lamb immune response data are plotted together in Figures 4–6). Thus the peak output of T cell blasts, and in particular CD4⁺ blasts, occurred on day 3 in the previously infected lambs and was very similar to the T cell response of the adult sheep (Figure 4). A minor difference was observed in the CD8⁺ response in the previously infected group. The adult sheep showed a slight CD8⁺ blast cell response at day 3, as opposed to the lambs which did not; however, this difference was not statistically significant. A highly comparable T cell response was observed for control adults and lambs for all cell surface markers analysed.

The B cell response of both previously infected and control lambs was also very similar to that observed in the older sheep (Figure 5). The IgA⁺ blast cell response in previously infected lambs initially rose at day 3, as with adults; however, the day 3 level was the peak of the response which declined after this, as opposed to the adult sheep in which the IgA⁺ blast cell output continued to rise until peaking on day 5, and then declining. This difference may explain why in the previously infected lambs the total IgA antibody in the gastric lymph initially rose in parallel with observations in adults, but then decreased again to pre-challenge levels by day 10 while the adult antibody levels remained high (Figure 6). However, parasite specific IgA antibody increased to, and was sustained at, approximately the same level in both previously infected lambs and adults, and indeed appeared to start rising sooner in the group of lambs. The level of IgA in control animals

did not vary throughout the course of the experiments, and lambs almost always had a lower concentration of total IgA than adults.

Whereas little difference was observed between lambs and yearlings in the current set of experiments, an earlier set of trials conducted at this laboratory with a similar *Teladorsagia*/sheep model did reveal definite age effects (11). These differences are summarised in Table 2.

In the earlier studies previously infected 10 month sheep contained relatively fewer challenge worms, and a greater proportion of these were arrested than 4-month-old lambs which had received an identical immunising regime. This increased susceptibility of the previously infected lambs was associated with much weaker gastric lymph responses compared to their yearling counterparts (11).

Why was this age difference not reproduced in the current batch of trials, especially when all the experiments were done at the same laboratory using similar techniques? Both sets of sheep were fed a maintenance diet and so different planes of nutrition should not have been a factor. It is possible that a change in sheep breed (formerly Greyface × Suffolk, now Leicester × Blackface or Suffolk × Dorset) could account for the difference, although no differences have been observed in the susceptibility of the Moredun flock to nematode parasites as a result of these breed changes. However, it seems most likely that a difference in the immunising regime offers the most plausible explanation. In the 1980s, 2000 *T. circumcincta* L3 were given to the previously infected sheep 5 days a week whereas in the recent series of trials this dose was administered only three times per week, i.e. the recent sheep received only 60% of the dose given in the 1980s. Exposure to the heavier immunising infection appeared to confer a more solid immunity to subsequent challenge in

Table 2 Age and immunity to *Teladorsagia circumcincta* in previously infected sheep: the effect of the size of the immunising infections on the response to challenge

Frequency of trickle doses ^a	Age of sheep	Degree of immunity 10 days post-challenge		Post-challenge gastric lymph response	Trial name	Reference
		% Protection ^b	% Arrested			
3 per week	Lambs	87.9	28.4	+++	Expt 5	This paper
		53.9	13.6	+++	Expt 6	
	Yearlings	66.3	31.0	+++	Expt 2	(10)
		77.9	38.2	+++	Expt 3	
5 per week	Lambs	16.8	22.0	+	Y1	(11)
		51.4	6.3	+	Y2	
	Yearlings	62.3	74.5	+++	O1	(5)
		89.6	80.0	+++	O2	

^a2000 L3 per dose for 8 weeks.

^b% Protection = $100 \times [1 - (\text{number of worms in immunised group} / \text{number of worms in control group})]$.

yearlings and yet make the lambs more susceptible (Table 2). There was no evidence from the recent trials with the lighter trickle infection to support the idea that one or more components of the immune response were defective in lambs. This includes examination of the abomasal histology where for example mast cell numbers were in the normal range (data being prepared for publication). We therefore hypothesise that only older, more resilient sheep were able to respond adequately following the heavier trickle, whereas the growing lambs, being less able to cope with the pathological effect of the greater parasite load, were only able to mount a weak, relatively ineffective response post-challenge.

In conclusion, we suspect that age and acquired immunity in ovine gastrointestinal nematodiasis is more likely to be due to the lack of resilience to infection on the part of lambs than to a specific immunological deficiency.

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5 Protective immunisation of calves against *Ostertagia ostertagi* using fourth stage larval extracts.

Aileen M Halliday and W David Smith (2010). *Parasite Immunol.* **32**: 656-663.

This paper is the first of 2 papers investigating the use of fourth stage larval extract as vaccine antigens to protect ruminants against abomasal nematodes. Adult sheep immunised against the blood sucking nematode *Haemonchus contortus* using proteins derived from the gut membrane of adult parasites develop significant immunity against subsequent homologous infection (Newton and Munn 1999, Smith 1999). When the same approach was attempted using antigens from adult *Ostertagia ostertagi* to vaccinate cows, or from adult *Teladorsagia circumcincta* to vaccinate sheep, adequate protection was not conferred against homologous challenge (Smith *et al* 2000, Smith *et al* 2001). However, when the antigens derived from adult *O. ostertagi* were used to vaccinate sheep, which were then challenged with *Haemonchus contortus*, the sheep showed protection against *H. contortus* (Smith *et al* 2000). This demonstrated that potentially the lack of protection against homologous challenge was not necessarily due to a difference in the composition of the antigen, but was postulated to be due to the fact that adult *O. ostertagi* and *T. circumcincta* are not blood feeders, and therefore are not exposed to the same level of host immunoglobulin as *H. contortus* which are obligate blood-feeders. It was further hypothesised that the larval stages of *O. ostertagi* and *T. circumcincta*, which undergo a period of rapid development within the gastric

pits of the abomasum, may be exposed to a much higher level of host serous exudate, containing immunoglobulin, than adults on the abomasal surface.

The vaccine trials reported in this paper describe the preparation of detergent soluble antigen from fourth larval stage *Ostertagia ostertagi*, and the subsequent vaccine trials carried out in calves.

Contribution to the work

The candidate was involved in all stages of experimental design and conduct, including infection of donor calves and collection of fourth stage larvae, antigen preparation, conduct of vaccine trials, post-mortem procedures and parasitological analysis, ELISA and data analysis. Results were presented at internal meetings. A patent is in place with the candidate named as co-inventor, number [PCT/GB2010/002298](#)



Protective immunization of calves against *Ostertagia ostertagi* using fourth stage larval extracts

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SUMMARY

ConA lectin was used to isolate glycoproteins from detergent extracts of fourth stage *Ostertagia ostertagi* larvae. This preparation contained proteins additional to those observed in a similar fraction prepared from adult *O. ostertagi*. Two vaccine trials were conducted with this preparation, and sub-fractions thereof, in groups of 6–8 worm-free calves. All groups were challenged with 50 000 *O. ostertagi* larvae 1 week after the final immunization, and protection was assessed by comparing the egg and worm counts of the immunized groups with their respective controls. Immunization with the *ConA*-binding antigen or its sub-fractions induced high titre serum antibody responses. In the first trial, the cumulative egg count of the group immunized with unfractionated antigen was 60% lower than the corresponding control value, and worm counts were 47% lower. In the second trial, the cumulative egg counts of the vaccinated groups ranged from 70% to 85% lower than the corresponding controls, with worm counts up to 64% lower. It was concluded that detergent-soluble, *ConA*-binding extracts prepared from *O. ostertagi* fourth stage larvae contained protective immunogens that were as effective as the best antigens published for *O. ostertagi* to date.

Keywords calves, *ConA* lectin, fourth stage larvae, membrane glycoproteins, *Ostertagia ostertagi*, protective antigens, *QuilA*

INTRODUCTION

Ostertagia ostertagi is the most economically important helminth parasite of cattle in temperate parts of the

world (1). As with all gastrointestinal nematodes of ruminants, ostertagiasis is controlled almost entirely by the use of anthelmintic drugs combined with pasture management. Unfortunately, reports of anthelmintic resistance in bovine gastrointestinal nematodes are becoming more frequent. For example, benzimidazole (BZ) resistance was identified in cattle in New Zealand (2) and South America (3), and macrocyclic lactone (ML) resistance in New Zealand (4,5), the Americas (6–9) and Europe (10–14). However, as no detailed surveys have been made, the extent of the problem remains unknown and probably underestimated. It is anticipated that anthelmintic resistance in cattle nematodes including *O. ostertagi* is likely to follow the pattern experienced with sheep, where it has reached serious proportions (15–18).

Alternative methods for controlling bovine ostertagiasis remain an attractive prospect, in part because of the threat of drug resistance and partly because of increasing consumer sensitivity to the possibility of chemical residues in meat and milk. One such possibility for control is by vaccination. Earlier attempts to do this, using either infection with irradiated larvae (19,20) or immunization with crude somatic or excretory/secretory products of the parasites (21,22), were not successful. More recently, promising results have been obtained using fractionated native excretory/secretory products of adult parasites, with reductions in faecal egg counts of up to 80% (23–27).

During recent years, substantial protection against the important blood-sucking ovine nematode, *Haemonchus contortus*, has been achieved by immunizing sheep with various antigens isolated from the intestinal membranes of adult parasites, a topic that has been reviewed extensively (28–30). When the same gut antigen approach was tested against *O. ostertagi*, some protection was conferred, but not at a level deemed to have practical potential (31). However, as the same *O. ostertagi* antigens cross-protected efficiently against *Haemonchus* in sheep, it was reasoned

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that the relative failure may have been because adult *O. ostertagi* are not blood feeders and do not ingest sufficient antibody for the gut antigen approach to be highly effective against them.

It was hypothesized that developing fourth stage (L4) *O. ostertagi* might be more vulnerable to this type of vaccination than adult parasites. L4s inhabit and damage the gastric glands, and as such are likely to be continuously exposed to inflammatory exudate. In addition, as the L4s grow very rapidly (32), increasing their mass approximately 20-fold in 10 days, they may be more sensitive to digestive interference than their slower metabolizing adult counterparts.

This study compares briefly the gel profiles of ConA-binding glycoproteins isolated from L4 and adult *O. ostertagi* with those of adult *H. contortus* and describes the outcome of two protection trials where the L4 preparation and sub-fractions made from it were evaluated as protective antigens in calves.

MATERIALS AND METHODS

Animals

All calves were reared and housed indoors in conditions designed to exclude accidental infection with nematode parasites.

Those used as donors for *O. ostertagi* eggs or fourth stage larvae were of various breeds and aged between 3 and 12 months at the time of infection. Those used in the vaccine trials were castrated Holstein–Friesian crosses aged 6–8 or 10–12 months in Experiments 1 and 2, respectively.

Parasites

Infective larvae were from strains of *O. ostertagi*, which have been maintained at Moredun Research Institute for several years.

Parasitological techniques

The methods for faecal egg counting and enumeration of worm burdens have been described before (33,34).

Fourth stage *O. ostertagi* larvae were harvested from donor calves that had been infected with a single dose of approximately 200 000 L3 7 days earlier. Soon after the animals had been killed by captive bolt and pithing, the abomasum were removed and the contents discarded. After a brief rinse in warm saline, each abomasum was pinned mucosal surface uppermost to a block of polystyrene, which was then inverted and floated in a large Baermann funnel containing warm saline. Following four hours at

37°C, fourth stage larvae were drained from the base of the funnel. The funnels were then incubated at 4°C overnight by which time any larvae still in suspension had settled out and could be drawn off. All larvae were frozen at –70°C until required for antigen extraction.

SDS–PAGE

Prior to SDS–PAGE, samples were heated at 100°C for 3 min in an equal volume of 63 mM Tris–HCl pH 6.8 containing 5% (w/v) SDS, \pm 10 mM DTT under non-reducing or reducing (10 mM DTT) conditions and separated on 4–12% gradient acrylamide gels (BIO–RAD, Hercules, CA, USA). Molecular weight markers (Fermantas, Burlington, Ontario, Canada) were run on each gel, and the gels were either stained with coomassie blue R250 (SIGMA, St Louis, MO, USA) (0.025% in 40% methanol/10% acetic acid) and destained in 20% methanol/10% acetic acid, or silver stained as follows. After SDS–PAGE, the gels were washed three times in distilled water and then fixed overnight in 40% methanol/10% glacial acetic acid. This was followed by incubation for 20 min in 20% methanol/5% acetic acid and then 4 \times 15 min washes in distilled water. Gels were then incubated in 50 mL 5 mg/L DTT for 45 min and then for 40 min in 50 mL 0.1% w/v AgNO₃, followed by two rapid washes in water and two washes in 25 mL 3% Na₂CO₃. The gels were then developed in 50 mL 3% Na₂CO₃ with the addition of 25 μ L formalin, and the development stopped after 15 min by adding 20 mL 2.3 M citric acid.

Immunoblotting

SDS–PAGE-separated proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA) using a semi-dry apparatus. Membranes were blocked in 10% Marvel (Premier Foods International, Spalding, Lincs., UK) in 10 mM Tris, 0.5 M NaCl, 0.05% (v/v) Tween-20, 0.02% (w/v) thimerosal (TNTT), the assay diluent and wash buffer, overnight at 4°C. Membrane strips were washed three times for 5 min in TNTT and then incubated with pooled serum samples from each group, diluted 1/300 in TNTT, for 2 h at room temperature. They were then washed in TNTT and incubated with rabbit anti-bovine immunoglobulin horseradish peroxidase-conjugated antibody diluted 1/1000 in TNTT (P0159; DAKOcytation, Glostrup, Denmark). After a further wash, the blot was developed using diaminobenzidine substrate (DAB; SIGMA-Aldrich, St. Louis, MO, USA), and the reaction stopped after 2 min by washing in several changes of distilled water.

Protein concentrations

These were estimated by the bicinchoninic protein assay reagent according to the manufacturer's instructions (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Preparation of immunogens

Ostertagia ostertagi L4s were homogenized in homogenizing buffer (PBS/1 mM EDTA/1 mM phenylmethylsulphonyl fluoride) using Lysing matrix C (MP Biomedicals, Irvine, CA, USA) for 45 s. Homogenized extracts were centrifuged for 2 min at $1000 \times g$ and then for 30 min at $25\,000 \times g$. The pellet was homogenized in buffer containing 0.1% (v/v) Tween 20 and centrifuged for 30 min at $25\,000 \times g$. The last step was repeated, and the pellet extracted in homogenizing buffer containing 2% (v/v) reduced Triton X-100 (SIGMA-Aldrich) without EDTA. The extract was centrifuged for 1 h at $100\,000 \times g$, and the supernatant was filtered (0.22 μ m). The Triton X-100 extracts of *O. ostertagi* L4s were then diluted fourfold with 10 mM Tris-HCl, 0.5 M NaCl, 0.05% NaN₃, 10 μ M MnCl₂ and 100 μ M CaCl₂, pH 7.4 (Lectin Wash Buffer, LWB). The solution was pumped (8 mL/h) at 4°C through Concanavalin A (ConA) lectin crosslinked to agarose beads (Vector Laboratories, Burlingame, CA, USA) contained in a column. After thorough washing in LWB/0.5% (v/v) reduced Triton X-100, the column was eluted with LWB/0.25% (w/v) CHAPS/0.2 M methylmannopyranoside/0.2 M methylglucopyranoside.

For elution, sufficient sugar solution was pumped onto each column to cover the beads, and then the flow was stopped for approximately 1 h. The pump was re-started, and the peak monitored at OD₂₈₀ was retained as the '1 h eluate'. The elution process was then repeated exactly, except that the flow was stopped overnight to produce an 'overnight eluate'. The eluates were pooled and passed through a column of Sephadex G-25 to remove the sugar and exchange the buffer to 10 mM Tris-HCl, 0.1% (w/v) CHAPS, pH 7.4, and stored at -70°C before using as immunogens.

For Experiment 2, the ConA eluate was further fractionated on a MonoQ anion exchange column, 1 mL bed volume (Pharmacia, Pfizer, Kent, UK) equilibrated in 10 mM Tris/0.1% (w/v) CHAPS pH 7.4. The ConA eluate was applied to the column (1 mL/min), and unbound proteins were collected. The bound proteins were eluted by a linear gradient increase in NaCl from 0 to 1 M over 20 mL, with 10×2 mL fractions being collected. These fractions were then pooled as follows: pool 1 = unbound material and proteins eluted with up to 0.1 M NaCl; pool 2 = frac-

tions eluted between 0.1 and 0.5 M NaCl; and pool 3 = fractions eluted between 0.5 and 1.0 M NaCl.

ELISA for antibodies to immunogens

Microtitre plates were coated overnight at 4°C with 50 μ L coating protein per well (ConA eluate), at 0.5 μ g/mL in 50 mM sodium bicarbonate buffer, pH 9.6. The plates were washed six times with wash buffer (PBS, 0.05% v/v Tween-20), then blocked with 200 μ L 10% (w/v) infasoy (Cow and Gate, Trowbridge, Wiltshire, UK) in TNTT overnight at 4°C. After washing, 50 μ L serum per well, diluted 1 : 2000 in TNTT, were added for 1 h at room temperature. The wells were re-washed and, 50 μ L peroxidase-conjugated rabbit anti-cow immunoglobulin diluted 1 : 1000 in TNTT added for 1 h at room temperature. After a final wash, 50 μ L *o*-phenylenediamine dihydrochloride substrate (Sigma) was added to each well. After 10 min in the dark, the colour reaction was stopped by the addition of 25 μ L 2.5 M sulphuric acid per well and OD values read at 490 nm. Each test sample was assayed in triplicate. Pooled serum taken at the time of challenge from the group of calves in Experiment 2 immunized with the unfractionated ConA eluate was included on each plate as a reference sample, and OD values expressed relative to this value.

Design of protection experiments

Two immunization-challenge trials were conducted with weight-balanced groups of calves. Trial 1 contained 15 calves, and 33 calves were used in Trial 2. All groups were immunized three times at 3-week intervals and challenged with 50 000 *O. ostertagi* L3 1 week later. Immunogens were diluted with cold phosphate-buffered saline, pH 7.4, (PBS) and mixed with QuilA (Superfos Biosector) so that each calf received either 20 mg (Experiment 1) or 5 mg (Experiment 2) of adjuvant at each immunization. Control immunogen was prepared identically, except that PBS was substituted for antigen, and administered to all challenge control animals. In Trial 1, seven calves were immunized with 95 μ g of antigen on each vaccine day (Group 1), and eight controls were given QuilA alone (Group 2). In Trial 2, seven calves were injected with 50 μ g unfractionated ConA eluate and acted as positive controls (Group 1). Group 2 (seven calves) were immunized with 16 μ g of the Pool 1 preparation. Group 3 (six calves) received 19 μ g of Pool 2 and Group 4 (six calves) were given 14 μ g of Pool 3 preparation on each occasion. Group 5 (seven calves) received adjuvant alone and served as the challenge controls. One ml of immunogen was injected intramuscularly into each side of the neck on each vaccine day. All animals

were bled at approximately weekly intervals to monitor the kinetics of the antibody response.

Statistical methods

Arithmetic group means are shown throughout with the standard errors of the mean. Significant differences between groups were calculated by the *t* test in Experiment 1 and by analysis of variance followed by Tukey's test in Experiment 2. To satisfy Bartlett's test for equal variances, the egg data in Experiment 2 were log transformed prior to analysis.

RESULTS

Yield of fourth stage larvae and ConA-binding membrane proteins

Recovery of fourth stage *O. ostertagi* larvae from donor calves ranged from 5% to 20% of the dose given. The yield of ConA-binding membrane proteins was approximately 0.3 mg per 100 000 fourth stage larvae.

Comparison of L4 and adult ConA-binding proteins

ConA-binding integral membrane proteins, prepared in the same way from adult *H. contortus* or fourth and adult stages of *O. ostertagi*, were compared by gel analysis and western blotting. Coomassie-stained gels indicated differences in the profiles of all three fractions (Figure 1a), although additional bands present in the L4 but not in the adult *O. ostertagi* preparations were of most interest in this case.

When the three ConA-binding fractions were probed with anti-sera from calves that had been immunized with material obtained in the same way from adult *O. ostertagi*, additional bands were still detected in the L4 fraction (Figure 1b).

Digesta from a worm-free calf was treated in exactly the same way as the L4s, but no protein peak was detected when the ConA column was eluted with sugar.

Protective capacity of detergent-soluble ConA-binding fraction from *O. ostertagi* L4s

Experiment 1: Immunization with the ConA lectin-binding fraction

Antigen used for immunization The gel profile of the preparation used to immunize the vaccinated calves in Trial 1 was very similar to that shown in Figure 1 lane 3.

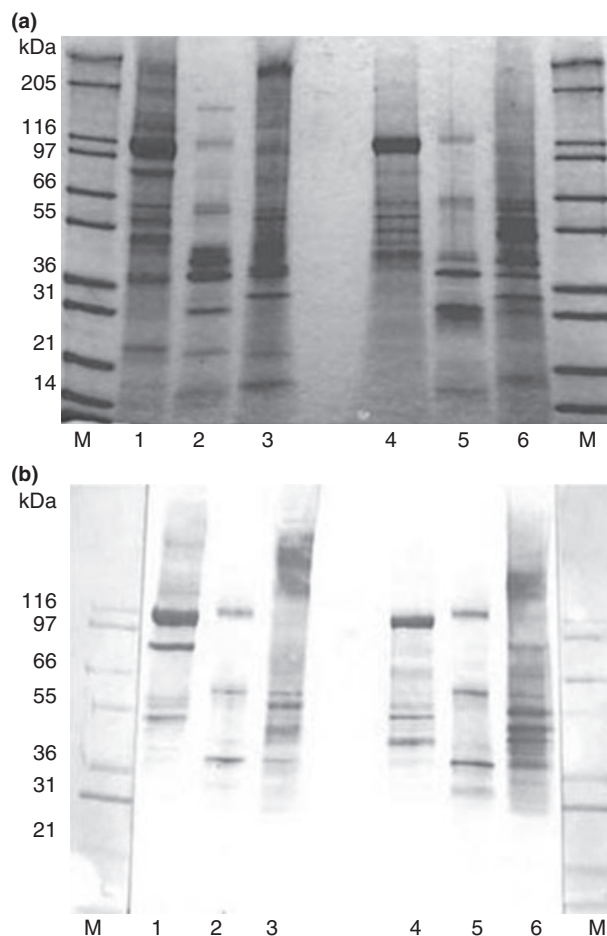


Figure 1 SDS-PAGE and immunoblot analysis of ConA binding membrane proteins from *Haemonchus contortus* and *Ostertagia ostertagi*. (a) Coomassie blue stained. (b) Immunoblot probed with sera from calves immunised with ConA binding membrane proteins from adult *O. ostertagi* (31). M, molecular weight markers. Lanes 1 and 4, adult *H. contortus*; lanes 2 and 5, adult *O. ostertagi*; lanes 3 and 6, L4 *O. ostertagi*. Lanes 1–3, non reducing SDS-PAGE. Lanes 4–6 reducing SDS-PAGE.

Antibody response Serum antibody titres in the control group remained at background concentrations throughout (Figure 2). In contrast, an increase in antibody titre was observed in the vaccinated group by week 5, 2 weeks after the second vaccination. This response reached a peak on week 8, 2 weeks after the third immunization.

Egg and worm counts Mean egg counts of the immunized calves were lower than that of the controls throughout the experiment, although the difference was not statistically significant on Days 28 and 30 (Figure 3a). However, the group means of the cumulative eggs per gram over Days 19–30 were significantly different ($P = 0.01$), with the vaccinated animals shedding 60% fewer eggs.

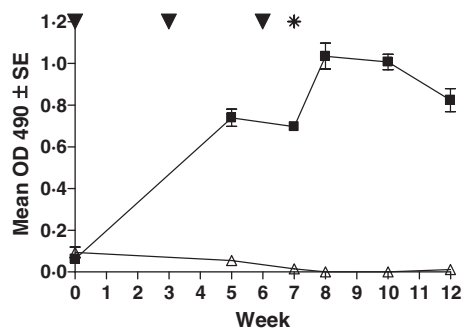


Figure 2 Kinetics of the antibody response to *O. ostertagi* L4 ConA binding extract, monitored using ELISA, following vaccination of calves with *Ostertagia ostertagi* L4 antigens or adjuvant alone in Experiment 1. Closed squares = vaccinates; open triangles = controls. Closed triangles = vaccinations. Star = challenge.

The mean number of worms recovered from the vaccinated animals was 1909 ± 252 , significantly ($P < 0.01$) fewer than from the controls that contained 3621 ± 414 (Figure 3b). The sex ratio of the worms recovered from each group was also different. The percentage of male worms recovered from the vaccinates (20.0 ± 3.5) was significantly ($P < 0.02$) lower than from the controls (37.4 ± 5.3). Small numbers of early fourth stage larvae were also found in some calves, but no difference between vaccinates and controls was observed.

Experiment 2: Immunization with sub-fractions of the ConA lectin-binding fraction

This trial was carried out to determine whether the level of protection detected in the first experiment could be improved if fractions more enriched for the protective components were prepared.

Antigens used for immunization The SDS-PAGE profiles of the fractions used to immunize the three vaccinated groups in trial 2 are shown in Figure 4.

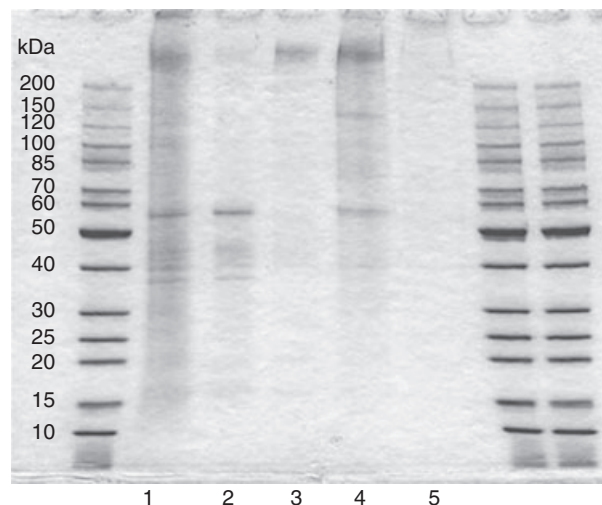


Figure 4 Non-reducing SDS-PAGE of the preparations used as antigens in Experiment 2. M, molecular weight markers. Lane 1 = ConA binding fraction; lane 2 = Pool 1, samples represented by lanes 3 and 4 were pooled to generate Pool 2, lane 5 = Pool 3.

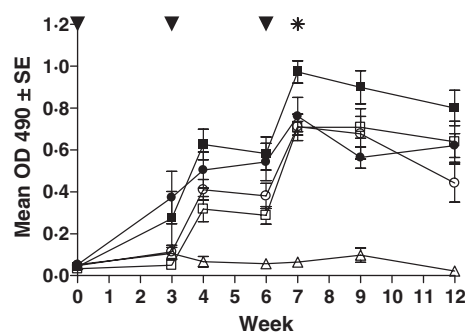


Figure 5 Kinetics of the antibody response to *O. ostertagi* L4 ConA binding extract, monitored using ELISA, following vaccination of calves with *Ostertagia ostertagi* L4 fractions or adjuvant alone in Experiment 2. Closed squares = group 1. Open squares = group 2. Closed circles = group 3. Open circles = group 4. Open triangles = group 5 (controls). Closed triangles = vaccinations. Star = challenge.

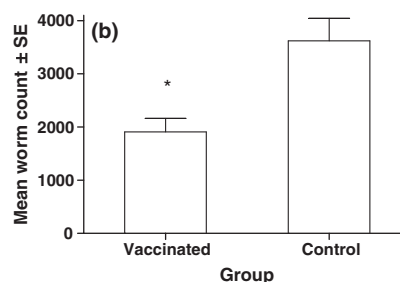
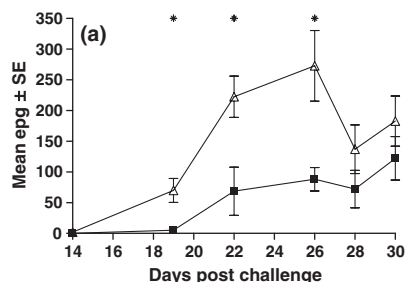


Figure 3 Parasitology data from Experiment 1. (a) Group mean faecal egg counts of vaccinated and control calves. Closed squares = vaccinated animals; open triangles = controls. (b) Group mean worm counts of vaccinated and control calves. * $P < 0.05$.

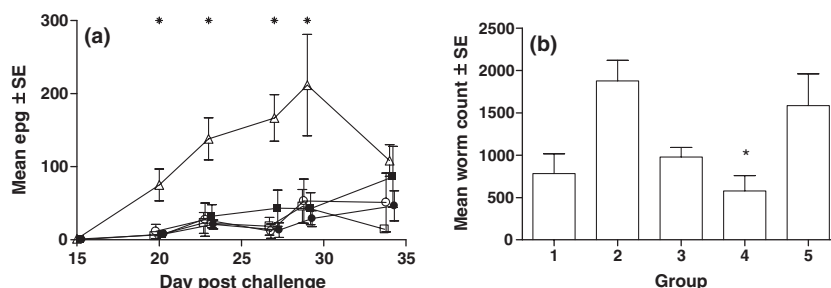


Figure 6 Parasitology from Experiment 2. (a) Group mean faecal egg counts of vaccinated and control calves in Experiment 2. Closed squares = group 1. Open squares = group 2. Closed circles = group 3. Open circles = group 4. Open triangles = group 5 (controls). (b) Group mean worm counts of the calves in Experiment 2. * $P < 0.05$ relative to the adjuvant only control group.

Table 1 Mean per cent protection data for cumulative egg counts and worm burdens, Trial 2

Group	Antigen	% Protection (eggs per gram)*			% Protection (worm burden)*		
		Mean	SE	<i>P</i> value	Mean	SE	<i>P</i> value
1	ConA	70	13	<0.05	51	15	ns
2	Pool 1	85	6	<0.01	–18.5	15	ns
3	Pool 2	83	5	<0.05	38	7	ns
4	Pool 3	78	11	<0.05	64	11	<0.05

*Relative to the adjuvant only control group.

Antibody response The kinetics of the antibody responses of each group is shown in Figure 5. All vaccinated groups showed a similar antibody response to *O. ostertagi* L4 antigen and had significantly ($P < 0.01$) higher antibody titres compared to the control group from 1 week after the second immunization until the end of the experiment.

Egg and worm counts All vaccinated groups showed significantly reduced egg counts compared to the adjuvant only control group from Day 20 to Day 29 (Figure 6a). The group means of the cumulative eggs per gram over Days 19–34 were significantly different for each of the vaccinated groups compared to the controls, with the corresponding percentage protection ranging from 70% to 85% as detailed in Fig 1. Only Group 4 showed a significant reduction in worm burden at necropsy, with 64% fewer worms than the control group (Figure 6b and Table 1). The percentage of male worms recovered from each group vaccinated with subfractions of the ConA lectin-binding fraction was significantly ($P \leq 0.05$) lower than from the controls.

DISCUSSION

This study described the preparation of Triton-soluble extracts from L4 *O. Ostertagi* containing multiple glyco-

proteins, compared these extracts briefly with similar preparations derived from adult *O. ostertagi* and *H. contortus* and then detailed two vaccine trials carried out using the L4 extract. *O. ostertagi* larvae were obtained by culling infected calves 7 days post infection, at which time point previous studies have shown the larvae to be at the L4 stage of development (32,35).

SDS-PAGE analysis showed the presence of additional protein bands in the L4 extract compared to the two adult extracts. As it was not possible to obtain the L4s without some contaminating digesta, the possibility existed that plant material was the source of some of the additional protein bands; however, this possibility was discounted when attempts to make a similar preparation from worm-free abomasal digesta did not yield any protein, possibly because of the cellulose cell walls of the plant cells, which make up the bulk of the digesta being resistant to Triton extraction.

Another possibility was that some of the polypeptides detected in the L4 preparation, which were additional to those observed in similar preparations from adult worms, were bovine in origin – perhaps from small pieces of abomasal tissue leaching from the mucosa when it was being incubated at 37°C to recover the larvae. However, an immunoblot developed with serum from calves immunized with adult *O. ostertagi* proteins revealed that several of these bands could not have been bovine proteins. Cross-reactivity of the L4 bands to anti-sera raised against adult extract was possibly because of similar proteins being present or cross-reactive glycans present on different proteins.

The difference in protein profiles between the ConA-binding fraction of L4 and adult *O. ostertagi* Triton-soluble extracts raised the possibility that the polypeptides present in the L4 extract but not in the adult extract might be gut proteins transiently expressed in the rapid growth phase of the L4s. If so, it was speculated that this preparation might contain candidate protective antigens, which would be worth investigating in a protection trial.

The first trial showed that calves vaccinated with the L4 ConA-binding preparation shed 60% fewer *O. ostertagi* eggs and contained significantly fewer worms than controls immunized with adjuvant alone. This result was confirmed and extended in the second vaccine trial where the most protective sub-fraction of the same preparation reduced worm eggs by 85% and worm numbers by 64%. Each group of calves vaccinated with L4 extract sub-fractions in the second trial showed significantly reduced egg output; however, this was not always accompanied by a significant reduction in worm burden. It is possible that in these cases, the vaccine caused a reduction in fecundity of female worms or delayed maturation of the worms; however, further investigation would be required to determine the exact mechanisms at work in these cases.

Host-mediated inhibition of larval development at the early L4 stage is a phenomenon that can be observed in cattle that have acquired resistance through exposure to *O. ostertagi* (36,37); however, this manifestation of immunity did not appear to be present following vaccination with the L4 ConA-binding extract as no differences were observed in the number of early L4 stage larvae recovered between groups from the calves in Experiment 1.

Previous studies have repeatedly demonstrated the protective capacity of *H. contortus* extracts prepared in a similar manner (28–30). When sections of *H. contortus* were probed with serum from vaccinated sheep, the luminal surface of the intestines and the subcuticular muscle blocks were observed to be coated with host immunoglobulin (38). Therefore, it was hypothesized that the *O. ostertagi* extracts prepared for use in these trials may have contained gut membrane glycoproteins, amongst others. The finding that vaccination with the preparation from L4s was more protective than that from adults suggests that additional components were present in the L4 extract. If the preparation process is indeed extracting gut membrane proteins, this would imply that the proteins present on the L4 gut during the rapid growth phase may be different to those in the adult gut, rather than just more abundant. The trials described in this study resulted in greater protective effects than those observed earlier with equivalent preparations from adult worms (31) and as good as any protection achieved in published *O. ostertagi* vaccine trials (27). It was also interesting to note that these effects were achieved with doses of <20 µg protein per injection. Much work remains to identify the protective components within each fraction and to determine whether or not they are derived from the L4 intestinal cells; however, these results point to extracts derived from L4 *O. ostertagi* in this way representing possible vaccine candidates.

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DISCLOSURES

None.

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6 Attempts to immunise sheep against *Teladorsagia circumcincta* using fourth stage larval extracts.

Aileen M Halliday and W David Smith (2011). *Parasite Immunol.* **33**: 54-560.

This is the second of 2 papers describing vaccine trials carried out in ruminants using fourth stage larval extracts of abomasal nematodes.

As described previously, detergent soluble gut membrane proteins extracted from non blood-feeding nematodes such as *O. ostertagi* and *Teladorsagia circumcincta* do not confer significant protection against homologous challenge, yet do provide protection in sheep against heterologous challenge with the blood-feeding nematode *Haemonchus contortus* (Smith *et al* 2000, Smith *et al* 2001).

Following the success of vaccinating calves with detergent soluble antigen extracted from the fourth larval stage of *Ostertagia ostertagi*, it was hypothesised that the same approach may confer protection to sheep against *Teladorsagia circumcincta*, as the two parasites have a similar lifestyle including a period of rapid development within the gastric pit of the abomasum, the stage being targetted by a vaccine derived from L4 antigen.

The paper contained herein describes the preparation of antigen from the fourth larval stage of *Teladorsagia circumcincta*, and 2 vaccine trials carried out in sheep using the L4 antigen.

Contribution to the work

The candidate was involved in all stages of experimental design and conduct, including infectin of donor sheep and collection of fourth stage larvae, preparation of antigen for vaccine trials, carrying out vaccine trials, post-mortem procedures and parasitological analysis, ELISA and data analysis. Results were presented at internal meetings.

Attempts to immunize sheep against *Teladorsagia circumcincta* using fourth-stage larval extracts

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SUMMARY

A ConcanavalinA (ConA)-binding fraction of a detergent-soluble membrane extract from *Teladorsagia circumcincta* (formerly *Ostertagia circumcincta*) fourth-stage larvae was isolated, and two vaccine trials were conducted with this preparation in groups of 7 worm-free sheep. All groups were challenged with a total of 5000 *T. circumcincta* larvae from 1 week after the final immunization and protection assessed by comparing the egg and worm counts, and length of developing worms, of the immunized groups with their respective controls. Immunization with the ConA-binding antigen induced high-titre serum antibody responses in both trials. However, no significant reduction in either egg count or worm burdens was observed in the vaccinated groups in either trial. It was concluded that detergent-soluble, ConA-binding extracts prepared from *T. circumcincta* fourth-stage larvae did not contain significantly protective antigens, despite the fact that an extract prepared in a similar manner from *Ostertagia ostertagi* had previously significantly protected calves against homologous challenge.

Keywords ConA lectin, fourth-stage larvae, membrane glycoproteins, sheep, *Teladorsagia circumcincta*.

INTRODUCTION

Teladorsagia circumcincta is a parasitic nematode, which inhabits the abomasum of sheep. It is an important parasite in temperate regions of the world, in terms of both animal welfare and loss of productivity. Current control methods rely on the use of anthelmintic drugs combined with pasture management. However, alternative methods

of control are being sought, driven by concerns over potential residues in the food chain and increasing levels of parasite resistance to the drugs, isolates of *T. circumcincta* having been identified, which show phenotypic resistance to several classes of anthelmintic (1–7). As sheep do acquire protective immunity to *T. circumcincta*, which is associated with a significant local immune response, resulting in reduction in worm burdens, egg counts and worm lengths (8–16), one possibility for an alternative control method is vaccination.

There has been substantial success in vaccinating sheep against the blood-sucking nematode *Haemonchus contortus* using antigens derived from the intestinal gut membrane of adult parasites (17–19). When the same vaccine approach, using adult *Ostertagia ostertagi* as the starting material, was tested in calves, significant protection was achieved against homologous challenge, though not at a level deemed to be useful (20). As the same *Ostertagia* fraction was highly efficacious for sheep challenged with *Haemonchus*, it was hypothesized that adult *Ostertagia* do not ingest sufficient amounts of host antibody for the approach to be highly effective (21). However, when the same extraction process was used on fourth-stage *O. ostertagi* larvae, as opposed to adults, the resulting antigen did confer significant protection against homologous challenge (22). *O. ostertagi* undergoes a period of rapid growth in the gastric pits following host ingestion (23), during which time the fourth-stage larvae inhabit and damage the gastric glands. It was hypothesized that because of this damage, they are continually bathed in host inflammatory exudate some of which they ingest. Hence, their intestines are exposed to a greater dose of host immunoglobulin than those of their adult counterparts, which reside mainly on the mucosal surface. In addition, the rapid growth phase may make the fourth-stage larvae more sensitive to digestive interference than adult worms, hence the success of the vaccine.

Previous attempts to vaccinate sheep against *T. circumcincta* have proven largely unsuccessful (21,24–26); however, these experimental vaccines have been based on

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antigens derived from third-stage larvae or adult parasites. *T. circumcincta*, like *O. ostertagi*, undergo a rapid growth period in the gastric pits of the host following ingestion (23). Therefore, it was hypothesized that *T. circumcincta* might also be more vulnerable following host vaccination with L4-derived antigen.

This paper describes the outcome of two vaccine trials in which the ConcanavalinA (ConA)-binding fraction of detergent extracts of *T. circumcincta* fourth-stage larvae was evaluated as protective antigens in sheep.

MATERIALS AND METHODS

Animals

All sheep were Scottish Mule (Blackface ewe x Blue-faced Leicester tup) castrated males, reared and housed indoors in conditions designed to exclude accidental infection with nematode parasites. The first trial used 14 sheep and the second trial 42 sheep. All animals were 4 months old at the start of the experiments.

Infective larvae

Infective L3 were from an anthelmintic-susceptible *T. circumcincta* isolate, which had been passaged through sheep at Moredun Research Institute for a number of years. The L3 were stored at 4°C for up to 1 month prior to administration. All challenge doses used within each experiment were derived from the same batch of larvae.

Post-mortem procedures

Post-mortem procedures were carried out as described previously (10).

Parasitological techniques

The methods for faecal egg counting, enumeration of worm burdens and measuring worm length (n = at least 20 per sheep) were carried out as previously described (10,27).

Fourth-stage *T. circumcincta* larvae for antigen preparation were harvested from donor sheep, which had been infected with a single dose of approximately 200 000 L3 7 days earlier. Soon after the animals had been killed, the abomasas were removed and the contents discarded. After a brief rinse in warm saline, each abomasum was pinned with the mucosal surface uppermost to a block of polystyrene, which was then inverted and floated in a large Baermann funnel containing warm saline. Following 4 h at 37°C, fourth-stage larvae were drained from the base of

the funnel. The funnels were then incubated at 4°C overnight, by which time the larvae still in suspension had settled out and could be drawn off. All larvae were frozen at -70°C until required for antigen extraction.

Preparation of antigen

Teladorsagia circumcincta fourth-stage larvae (L4s) were homogenized in buffer [Phosphate buffered saline (PBS)/1 mM Ethylenediaminetetraacetic acid (EDTA)/1 mM phenylmethylsulphonyl fluoride] for 45 s using Lysing matrix C (MP Biomedicals, Irvine, CA, USA) in a precllys 24 machine (Bertin Technologies, Montigny-le-Bretonneux, France). During preparation of the antigen for Trial 2, protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) (500 µL/100 000 larvae) was also added. Homogenized extracts were centrifuged for 2 min at 1000 × g and then for 30 min at 25 000 × g . The pellet was homogenized in buffer containing 0.1% (v/v) Tween 20 and centrifuged for 30 min at 25 000 × g . The last step was repeated, and the pellet extracted in homogenizing buffer containing 2% (v/v) reduced Triton X-100 (Sigma-Aldrich) without EDTA. The extract was centrifuged for 1 h at 100 000 g , and the supernatant filtered (0.22 µm). The Triton X-100 extracts of *T. circumcincta* L4s were then diluted fourfold with 10 mM Tris-HCl, 0.5 M NaCl, 0.05% NaN₃, 10 µM MnCl₂, 100 µM CaCl₂, pH 7.4 (Lectin Wash Buffer, LWB). The solution was pumped (8 mL/h) at 4°C through ConA lectin cross-linked to agarose beads (Vector Laboratories, Burlingame, CA, USA) contained in a column. After thorough washing in LWB/0.5% (v/v) reduced Triton X-100, the column was eluted with LWB/0.25% (w/v) CHAPS/0.2 M methylmannopyranoside/0.2 M methylglucopyranoside.

For elution, sufficient sugar solution was pumped onto each column to cover the beads, and then the flow was stopped for approximately 1 h. The pump was re-started, and the peak, monitored at OD₂₈₀, was retained as the '1-hour eluate'. The elution process was then repeated exactly, except that the flow was stopped overnight to produce an 'overnight eluate'. The eluates were pooled and passed through a column of Sephadex G-25 to remove the sugar and exchange the buffer to 10 mM Tris-HCl, 0.1% (w/v) CHAPS, pH 7.4 and stored at -70°C before use as immunogens.

SDS-PAGE

SDS-PAGE was carried out using NuPAGE 4–12% Bis-Tris mini gels (Invitrogen, Life Technologies, Carlsbad, CA, USA) under reducing conditions, as per the manufacturer's instructions. Molecular weight markers (Fermentas,

Burlington, ON, Canada) were run on each gel, and the gels were stained with Simply Blue Stain (Invitrogen) and destained using distilled water.

Protein concentrations

These were estimated by the bicinchoninic protein assay reagent according to the manufacturer's instructions (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA).

ELISA for antibodies to antigen

Microtitre plates were coated overnight at 4°C with 50 µL coating protein per well (ConA eluate), at 1 µg/mL in 50 mM sodium bicarbonate buffer, pH 9.6. The plates were washed six times with wash buffer (PBS, 0.05% (v/v) Tween-20), then blocked with 200 µL 10% (w/v) infasoy milk powder (Cow and Gate, Trowbridge, Wiltshire, UK) in 10 mM Tris, 0.5 M NaCl, 0.05% (v/v) Tween-20, 0.02% (w/v) thimerosal (TNTT) overnight at 4°C. After washing, 50 µL serum per well, diluted 1 : 1000 in TNTT, were added for 1 h at room temperature. The wells were re-washed, and 50 µL peroxidase-conjugated monoclonal anti-sheep IgG (GT-34, Sigma-Aldrich) diluted 1 : 10 000 in TNTT added for 1 h at room temperature. After a final wash, 50 µL *o*-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) was added to each well. After 10 min in the dark, the colour reaction was stopped by addition of 25 µL 2.5 M sulphuric acid per well, and OD values read at 490 nm. Each test sample was assayed in triplicate. Pooled serum taken at the time of challenge from the group of sheep in Trial 1 immunized with the ConA eluate was included on each plate as a reference sample, and OD values expressed relative to this value.

Design of protection experiments

Two immunization-challenge trials were conducted with weight-balanced groups of sheep. Trial 1 contained 14 sheep, and 42 sheep were used in Trial 2. All groups were immunized three times at 3-week intervals. In Trial 1, both groups were challenged with 5000 *T. circumcincta* 1 week after the third immunization. In Trial 2, Groups 1–4 were challenged with 5000 *T. circumcincta* L3 1 week after the third immunization, and Groups 5 and 6 received 500 L3/day for 10 days, starting 1 week after the third immunization. Immunogens were diluted with 1 mL cold phosphate-buffered saline, pH 7.4, (PBS) and mixed with an equal volume of QuilA (Superfos Biosector, Frederikssund, Denmark) so that each sheep received 5 mg of adjuvant at each immunization. Control immunogen was prepared identically, except that PBS was substituted for antigen.

In Trial 1, seven sheep were immunized with 34 µg of antigen on each vaccine day (Group 1) and 7 controls were given QuilA alone (Group 2). In Trial 2, Groups 1, 3 and 5 were injected with 48 µg ConA eluate and Groups 2, 4 and 6 injected with adjuvant alone as respective control groups. One milliliter of immunogen was injected intramuscularly into each back leg on each vaccine day. All animals were bled at approximately weekly intervals to monitor the kinetics of the antibody response. In Trial 1, all animals were killed 35 days after challenge. In Trial 2, Groups 1 and 2 were killed 10 days after challenge, and Groups 4–6 were killed 28 days post-challenge (Table 1).

Statistical methods

Arithmetic group means are shown throughout with the standard errors of the mean. Analysis of variance

Table 1 Experimental design and rationale

Trial	Group	Antigen	Challenge ^a (day 0)	Trickle infection ^b (post-day 0)	Kill day	Purpose
1	1	+	+	–	35	Does vaccination protect against bolus challenge?
	2	–	+	–	35	Control for Group 1
2	1	+	+	–	10	Is worm growth stunted in vaccinated animals 10 days after bolus challenge?
	2	–	+	–	10	Control for Group 1
	3	+	+	–	28	Does vaccination protect against bolus challenge?
	4	–	+	–	28	Control for Group 3
	5	+	–	+	28	Does vaccination protect against trickle challenge?
	6	–	–	+	28	Control for Group 5

^a5000 infective L3; ^b500 infective L3 per day, for 10 days.

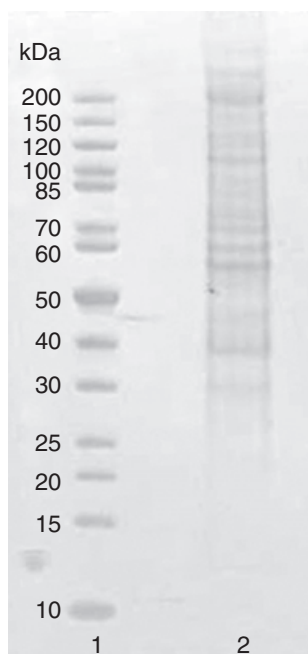


Figure 1 SDS-PAGE analysis of Concanavilin A (ConA)-binding extract from fourth-stage *Teladorsagia circumcincta*, reducing conditions. Lane 1, molecular weight markers. Lane 2, ConA-binding proteins.

(ANOVA) and Student's *t*-tests (two-tailed) were used to calculate stated probabilities. *T*-tests were used for the analysis of data from Trial 1 and Trial 2 Groups 1 and 2 and for comparison of pooled egg output data with that obtained previously in *O. ostertagia* trials in cattle (22). ANOVA was used for the analysis of data from Trial 2 Groups 3–6.

RESULTS

Yield of fourth-stage larvae and ConA-binding extract

Recovery of fourth-stage *T. circumcincta* larvae from donor sheep was approximately 30% of the dose given. The yield of ConA-binding proteins was approximately 0.23 mg per 100 000 fourth-stage larvae.

SDS-PAGE of ConA-binding extract

The ConA-binding extract used in Trial 2 was analysed by SDS-PAGE followed by coomassie staining, under reducing conditions (Figure 1). Proteins were observed at a wide range of molecular weights.

Antibody response

Serum antibody titres in the control groups of each trial remained at background concentrations throughout (Figure 2a,b). In contrast, an increase in antibody titre was observed in the vaccinated groups by week 4 (Trial 1) and week 5 (Trial 2), occurring after the second vaccination. This response reached a peak on week 7, 1 week after the third immunization. Analysis of variance of the antibody titres within the vaccinated groups in Trial 2 showed a significant difference at week 5 ($P = 0.029$); however, at all other times, there was no significant difference.

Egg and worm counts

No significant differences were observed in cumulative egg counts over the course of the trial (Figure 3a,b),

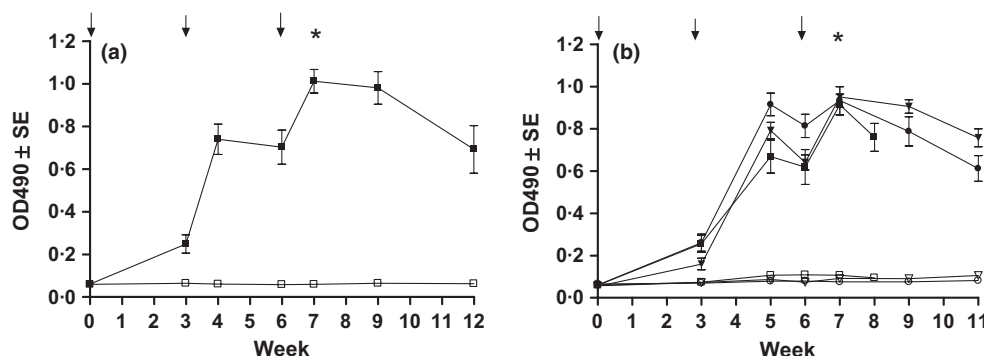


Figure 2 Kinetics of the serum IgG response to *Teladorsagia circumcincta* L4 Concanavilin A-binding extract, monitored using ELISA, following vaccination of sheep with *T. circumcincta* L4 antigens or adjuvant alone. (a) Trial 1: closed squares = vaccinates; open squares = controls. (b) Trial 2: closed squares = Group 1 (vaccinates killed 10 days post-challenge (dpc), bolus challenge); open squares = Group 2 (control group for Group 1); closed triangles = Group 3 (vaccinates killed 35 dpc, bolus challenge); open triangles = Group 4 (control group for Group 3); closed circles = Group 5 (vaccinates killed 35 dpc, trickle challenge); open circles = Group 6 (control group for Group 5). Downwards arrows = vaccinations. Star = challenge.

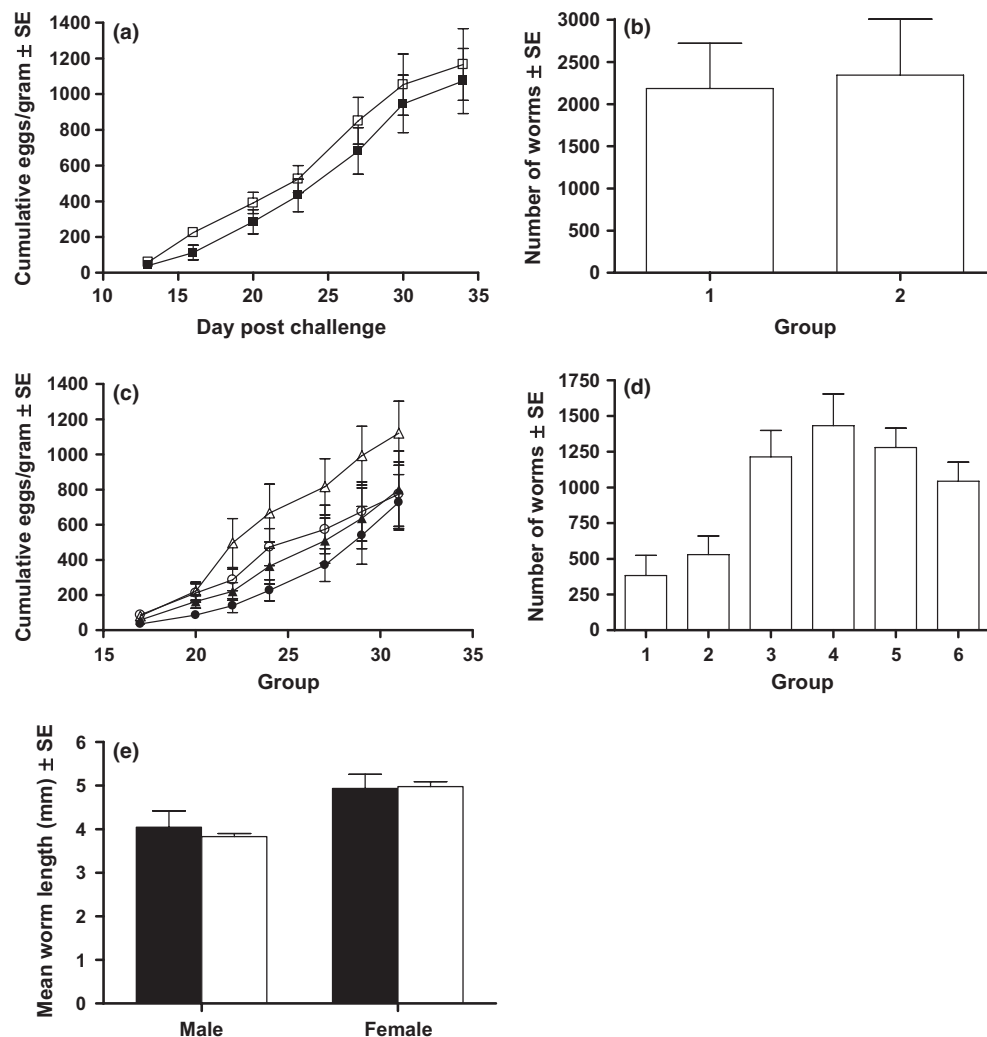


Figure 3 Parasitology data. (a) Trial 1 cumulative egg counts: closed squares = vaccinates; open squares = controls. (b) Trial 1 worm burdens: Group 1 = vaccinates. Group 2 = controls. (c) Trial 2 cumulative egg counts: closed triangles = Group 3 (vaccinates killed 35 dpc, bolus challenge); open triangles = Group 4 (control group for Group 3); closed circles = Group 5 (vaccinates killed 35 dpc, trickle challenge); open circles = Group 6 (control group for Group 5). (d) Trial 2 worm burdens. (e) Trial 2 worm lengths: black bars = Group 1 (vaccinates killed 10 dpc, bolus challenge); white bars = Group 2 (control group for Group 1).

total worm burdens at necropsy (Figure 3c,d) or worm lengths (Figure 3e) between the vaccinates and their respective control animals in either trial. At the end of Trial 2, Group 3 showed a reduction of 29% in cumulative egg counts compared to Group 4; however, this reduction was not significant ($P = 0.28$). In Trial 2, egg counts were carried out on Groups 3–6 only, as Groups 1 and 2 were killed before the prepatent period. Comparison of the pooled egg output data from the vaccinated animals in Trial 1 (Group 2) and the Trial 2 Group 3 vaccinated sheep with the egg output data obtained from vaccinated animals in the previously published *O. ostertagia* trials showed a statistical differ-

ence in percentage protection between the species ($P = 0.003$).

DISCUSSION

This paper described the preparation of ConA-binding, detergent-soluble extracts from *T. circumcincta* fourth-stage larvae and detailed two vaccine trials carried out in sheep using the L4 extract. *T. circumcincta* larvae were harvested from the abomasa of sheep culled 7 days post-infection, at which time point the majority of the larvae are deemed to be at the L4 stage of development (23).

The rationale behind this choice of vaccine antigen was based on the success of recent vaccine trials against *O. ostertagi* in calves (22). Antigen for the two *O. ostertagi* trials was prepared from fourth-stage larvae in a manner similar to that used in the trials described herein and conferred significant protection upon the calves with reductions in egg counts of 60% and 70% and reductions in worm burdens of 47% and 51%, respectively. It was hypothesized that the success of vaccinating with L4 antigen, compared to adult antigen, was due to fourth-stage larvae being bathed in, and ingesting, serous exudate while undergoing their rapid growth phase in the gastric pits. This possibly makes them more sensitive to digestive interference by host immunoglobulin compared to adult parasites residing on the surface of the abomasum. *O. ostertagi* and *T. circumcincta* parasites have both been shown to contain host immunoglobulin in their gut (28), and *T. circumcincta*, like *O. ostertagi*, undergoes a period of rapid growth in the gastric gland upon entering the host abomasum (23). Both *O. ostertagi* and *T. circumcincta* (originally placed in the genus *Ostertagia*) are members of the subfamily *Ostertagiinae* (family Trichostrongylidae) (29–31). Aside from differing host specificity, they have similar life cycles, with a free-living stage from eggs to L3, followed by a change to parasitic behaviour upon ingestion by the ruminant host and a predilection for the abomasal gastric pit as a growth site for continued development into adult parasites (32). Given these similarities between the two parasites and the success of the *O. ostertagi* trials (22), it was hypothesized that the *T. circumcincta* larvae may also be sensitive to digestive interference following host vaccination with L4 antigen.

The first trial involved 2 groups of animals, both of which were killed at 35 days after challenge. Egg counts taken from day 13 onwards, and worm counts at post-mortem, did not reveal any protection afforded to the vaccinated group. The same absence of protection, at day 28, was confirmed in the second trial following challenge with a single bolus of 5000 larvae. A reduction of 29% in final cumulative egg counts was observed in the vaccinated animals; however, this result was not statistically significant.

No protection was afforded to the group of vaccinated animals killed at day 10 either. This time point was chosen as it was hypothesized that if the theory that vaccine-induced antibodies interfere with the digestive processes was correct, then retardation would be expected to be most obvious at the end of the rapid growth phase, stunted worms having previously been observed in sheep with acquired immunity to *T. circumcincta* (10).

The trickle challenge group was included to determine whether vaccine-induced immunity would be more apparent if the challenge was administered as a trickle infection as occurs naturally. Perhaps the immune response would

be able to cope better with the same challenge dose spread over 10 days rather than as a single bolus? Another possibility was that the early arriving larvae in the trickle challenge would stimulate an anamnestic response, which would adversely affect later arriving worms. However, no differences were observed in egg or worm counts between the vaccinated animals and controls using this challenge protocol either.

SDS-PAGE analysis of the ConA-binding, detergent-soluble L4 antigen revealed a complex mixture of proteins at a wide range of molecular weights, with prominent bands at approximately 40, 60 and 200 kDa. The vaccinated groups of animals that were hyperimmunized with this preparation showed a secondary humoral immune response, with serum levels of IgG specific for the L4 antigen rising following the second immunization. However, as no significant reduction in worm burdens, egg counts or worm length was subsequently detected, this suggests that the immune response was either quantitatively or qualitatively insufficient to provide protection against the parasite. During preparation of the antigen for the second trial, broad-spectrum protease inhibitors were added to determine whether active proteases in the extract were interfering with immunogenicity. This, however, did not have any impact on serum antibody levels nor provide significant protection against the parasite.

It is interesting, and important, to compare the results of the current trials with the success obtained in the *O. ostertagi* trials in which substantial protection was provided to calves following immunization with an *O. ostertagi* L4 antigen prepared in a similar manner and homologous challenge (22). Comparison of the egg output data from sheep and cattle showed a statistical difference in percentage protection between the species. More importantly, from a biological point of view, the egg output of the vaccinated sheep in the *T. circumcincta* trials was not reduced by 60–80% as required for a biologically effective worm control strategy, whereas the vaccinated cattle in the *O. ostertagi* trials did attain this reduction (33). Comparative proteomics could provide some clues as to whether there are differences in the protein profile of the larval extracts or whether the explanation possibly lies in differences in the way the ovine and bovine immune system respond to either the parasites or this type of vaccination or both. Cross-protection studies against heterologous challenge may also provide more insight into this phenomenon.

In conclusion, this paper has described two vaccine trials in sheep using ConA-binding, detergent-soluble extracts of fourth-stage *T. circumcincta*, which did not provide protection against homologous challenge despite promising results obtained recently in similar *O. ostertagi*

trials. The exact reasons for the failure of the antigen to provide protective immunization remain to be elucidated.

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7 Transcriptional changes in *Teladorsagia circumcincta* upon encountering host tissue of differing immune status.

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As described in Paper 2 of this thesis (Halliday *et al* 2009), manifestations of immunity are observed as early as 48 hours post challenge in sheep which have previously been infected with *Teladorsagia circumcincta*. This suggests critical early host-parasite interactions occurring in the abomasum which determine whether or not the larvae can establish.

The aim of this paper was to expose infective 3rd stage *Teladorsagia circumcincta* to either naïve or immune host abomasal extract, then to carry out transcriptomic analysis on the parasites to identify genes which may be involved in larval infectivity and response to host immune status.

Transcriptomic sequencing was carried out using the Roche 454 platform, with sequencing and subsequent bioinformatic analysis carried out in conjunction with The GenePool facility at Edinburgh University.

Contribution to the work

The candidate was involved in all stages of experimental design and conduct, including preparation of larvae and abomasal environments, proteomic analysis of abomasal extracts, preparation of larvae for transcriptomic analysis and

bioinformatic analysis of results. Results have been presented at local, national and international meetings.

Transcriptional changes in *Teladorsagia circumcincta* upon encountering host tissue of differing immune status

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SUMMARY

The aim of this study was to elucidate transcriptional changes in the parasitic nematode *Teladorsagia circumcincta* upon encountering either naïve or immune ovine hosts. Pools of 100 000 exsheathed 3rd-stage *T. circumcincta* larvae were exposed *in vitro* to either an immune or naïve ovine abomasal environment, RNA was extracted from the larvae and sequenced using the Roche 454 platform. Each sample produced approximately 82 000 reads that assembled to give approximately 5500 Isotigs (contigs). The two sequence datasets were clustered together to give a total of 6969 clusters of which 18 were differentially expressed ($P < 0.001$) between the two groups. Clusters with a predominance of reads in larvae exposed to the immune abomasal environment encoded homologues of peptidyl-glycine alpha-amidating monooxygenase, heat shock-protein 16-2 and IDA-1, a tyrosine phosphatase-like receptor protein. Clusters with a predominance of reads in the naïve environment encoded homologues of cytochrome b, EGg Laying defective family member 21 and NADH dehydrogenase subunit 5. Gene ontology analyses indicated that larvae exposed to the immune environment showed an increase in expression of genes involved in 'carbon utilization', 'response to stimulus' and 'developmental process'. These data suggest that *T. circumcincta* modulates gene expression in response to the immune status of the host.

Key words: *Teladorsagia circumcincta*, gene expression, parasitic nematodes, sheep, proteomics, immunity.

INTRODUCTION

Teladorsagia circumcincta is a parasitic nematode that inhabits the abomasum of small domestic ruminants. It is an important parasite in temperate areas of the world, both in terms of animal welfare and economic loss. Animals suffer from diarrhoea, reduced growth, emaciation and high morbidity; the estimated cost to the British farming industry through gastrointestinal parasites alone is £84 million annually (Nieuhof and Bishop, 2005; Scott *et al.* 2007). Current control methods rely on pasture management and the use of anthelmintic drugs; however, the emergence of widespread resistance to anthelmintic treatment is driving the search for alternative methods of control (Sargison, 2011).

Sheep can acquire immunity to *T. circumcincta* following exposure, leading to investigation of vaccination as one such alternative method of control (Smith *et al.* 1983, 1986; Miller, 1996; Balic *et al.* 2003; Macalodowie *et al.* 2003; Halliday *et al.* 2009, 2010). Manifestations of immunity include a reduction in total worm burden and egg output, a reduction in developing worm length and a reduction in larval establishment in the abomasum of immune sheep,

through either exclusion or expulsion, within 48 h of ingestion (Stear *et al.* 1995, 1999a,b; Jackson *et al.* 2004; Halliday *et al.* 2007, 2009). This implies that there are critical events occurring very early in the host-parasite interaction that determine whether the parasite can establish in the abomasum of its ovine host.

The high capacity and relatively long read length of 454 sequencing technology (Margulies *et al.* 2005; Roche, 2011) has made it suitable for transcriptomic studies and several parasite species have been investigated in this way including *T. circumcincta*, *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Fasciola hepatica* (Young *et al.* 2003; Cantacessi *et al.* 2010a,b; Dicker *et al.* 2011). Here, we used 454 sequencing to examine gene expression in the infective 3rd larval stage of *T. circumcincta* immediately following exposure to either an immune or a naïve ovine host abomasal environment. By comparing gene expression in these environments we have identified a subset of the genes that may be important in larval infectivity and response to host immunity.

MATERIALS AND METHODS

Animals, post-mortem and generation of naïve and immune abomasal environments

All sheep used were Scotch Mules (Blackface ewe X Blue-faced Leicester tup) that were 8 months old at

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the start of the immunizing infection, and 10 months old at post-mortem. The sheep were born and raised in controlled parasite-free conditions to ensure that they remained parasite free until the time of trickle infection for the immunized group, and until the end of the experiment for the naïve group. Immunity to *T. circumcincta* was generated in 1 group of 6 sheep as detailed previously (Halliday *et al.* 2007). Briefly, a trickle infection of 2000 infective 3rd-stage larvae (L3) *T. circumcincta* was given 3 times per week for 8 weeks. This group of animals (immune) were then given Fenbendazole according to the manufacturer's instructions to remove the resident worms, followed 7 days later, by a single bolus challenge of 50 000 L3. Another group of 6 uninfected sheep (naïve) were maintained in parallel. Twenty-four hours after the bolus dose of larvae to the immune group, all the sheep were stunned with a captive bolt, exsanguinated, and the abomasum removed and opened along its lesser curvature. The abomasum was washed 3 times in warm saline, then two 2 cm² folds per sheep were removed, rinsed briefly with warm water to remove food debris and then incubated in 2 ml of warm Earle's Buffered Saline Solution (18 mM CaCl₂·2H₂O, 54 mM KCl, 8 mM MgSO₄·7H₂O, 1·16 M NaCl, 10 mM NaH₂PO₄, 56 mM glucose; EBSS) for 3 h at 37 °C, in 6-well cell culture plates. The abomasal fold sample was then removed and discarded, and the buffer retained as either a naïve or immune abomasal environment sample.

Ex-sheathing larvae and exposure to abomasal environment

Approximately 200 000 *T. circumcincta* L3 were washed in phosphate-buffered saline (PBS)/penicillin (250 IU/ml)/streptomycin (250 mg/ml)/gentamicin (312·5 µg/ml)/amphotericin B (5 µg/ml) (PBS wash solution), then centrifuged for 2 min at 200 g and the supernatant fraction discarded. This was repeated twice, then the larval pellet was resuspended in 50 ml of warm EBSS/penicillin (100 IU/ml)/streptomycin (100 mg/ml)/gentamicin (125 µg/ml)/amphotericin b (5 µg/ml) (EBSS exsheathing buffer). CO₂ gas was bubbled gently through the larval suspension for 15 min, before incubating the suspension at 37 °C with shaking (100 rpm) for 4 h. Then 100 µl was removed every hour to monitor larval exsheathing. After 4 h of incubation, the larval suspension was split into 2 equal volumes and centrifuged for 2 min at 200 g. The supernatant fraction was removed and the larval pellets were resuspended in 1·5 ml of naïve or immune abomasal environment sample, prepared as described above, then incubated in 6-well plates at 37 °C with shaking (100 rpm) for a further 4 h. A 100 µl sample was removed after 2 h and 4 h to monitor larval viability. After 4 h the larvae were

centrifuged for 2 min at 200 g. The supernatant fraction was removed, and 1 larval pellet for each treatment snap frozen in liquid nitrogen.

Preparation of cDNA and 454 sequencing

Total RNA from the 2 larval samples, i.e. 1 from naïve- and 1 from immune-abomasal environment exposed larvae was prepared using TRIzol reagent (GibcoBRL, Life Technologies) following the manufacturer's instructions. The extracted RNA was treated with DNase (Ambion) as per the manufacturer's instructions, and cleaned up using a Qiagen RNeasy Minelute kit, as per manufacturer's instructions. RNA integrity and yield was examined by agarose gel electrophoresis and using a NanoDrop ND-1000 UV spectrophotometer v.3.2.1 (NanoDrop Technologies, Wilmington, DE, USA). First strand cDNA was constructed using the SMARTerTM PCR cDNA synthesis kit (Clontech/Takara Bio, CA) following the manufacturer's instructions. An optimized PCR cycling protocol of 14 cycles was used to amplify full-length cDNAs using 5' PCR Primer II A with Advantage 2 PCR buffer and polymerase mix (Clontech/Takara), and the amplified cDNA cleaned up using Qiagen PCR purification kit as per manufacturer's instructions. A total of 5 µg cDNA for each sample was delivered to TheGenePool, Edinburgh University, for next generation sequencing using Roche-454 Titanium technology (Margulies *et al.* 2005; Roche, 2011).

Bioinformatic analysis of sequencing data

Sequence data were assembled using Newbler (v2.55) Assembler software (Roche, 2010). The read data from each population were assembled separately, as joint assembly of the 2 sets of sequencing data resulted in unrealistically long contigs that were unlikely to represent biological entities. Following assembly, the Isotig sequences representing putative transcripts were submitted to the Blast2GO server (Conesa *et al.* 2005; Conesa and Gotz, 2008; Gotz *et al.* 2008). This carried out Blastx searches using the NCBI non-redundant database (Blast ExpectValue 1.0E-03), InterProScan searches for functional annotation (Hunter *et al.* 2009) and Gene Ontology based annotation (Ashburner *et al.* 2000). Sequences were annotated to KEGG biological pathways within Blast2GO with the parameters E-Value-Hit-Filter 1.0E-06, Annotation CutOff 55, GO weight 5 (Kanehisa and Goto, 2000; Ogata *et al.* 1999). Clustering of the combined Isotig datasets was carried out using UCLUST (v3.0.617) (Edgar, 2010) optimized at 65% identity, searching against forward and reverse strand, with optimal global alignment selected. The reads contained within each cluster were enumerated using a Perl script

that calculated the total number of unique reads originating from each sample and thus the abundance of transcripts from each sample.

Statistical analyses

A 2×2 Fisher's Exact test was conducted to test for differences between the mean proportion of Isotigs representing naïve and immune groups for each functional classification as identified by KEGG and GO analysis. The estimated P -values for all functional groups under KEGG and GO classification systems were then adjusted using the False Discovery Rate (FDR) approach (Benjamini and Hochberg, 1995) to take into account multiple comparisons of treatments.

For the 454 next-generation sequencing data, the strategy used to identify clusters indicated that there was likely to be excess clustering or overdispersion in the data. However, with a single replicate for each combination of cluster and treatment, it was not possible to quantify this effect. Therefore an approach was adopted which combined information from 2 statistical models that together spanned the range of results that would arise from a more appropriate model. The first model is a conservative model with dispersion parameter set at a maximum possible value (3.65), estimated from a separate model which confounded any cluster or cluster by treatment effects with this dispersion parameter. The second model is a liberal model which assumes that there is no overdispersion in the data, and hence sets the value of dispersion parameter as 1. Details of this statistical approach were previously described by Dicker *et al.* (2011).

The FDR adjusted p -value (Benjamini and Yekutieli, 2005) for a cluster is the minimum FDR for which the observed difference between the mean expression level of naïve and immune groups would be accepted as statistically significant. The FDR-adjusted P values (P_F) from the two statistical models were then used to categorize each cluster into 1 of 3 classes: clusters with P_F values of less than or equal to 5% under the conservative model were of prime scientific interest; clusters with P_F values greater than 5% under the liberal model were unlikely to be of interest; other clusters were of indeterminate status, but could be prioritized for further work with respect to either set of P_F values. FDR-adjusted p -values are hereafter denoted by P_F .

All statistical analyses were carried out using the R software version 2.11.1 (R Development Core Team, 2010).

Proteomic analysis of abomasal environments

SDS-PAGE of naïve and immune abomasal environment samples was carried out using NuPAGE 4–12%

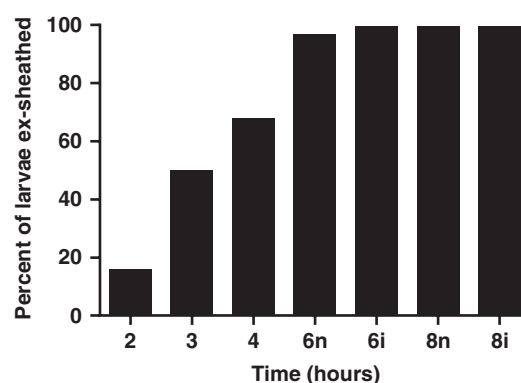


Fig. 1. Progress of larval exsheathing. 6-n and 8-n, larvae incubated in naïve abomasal environment sample. 6-i and 8-i, larvae incubated in immune abomasal environment sample.

Bis-Tris mini gels (Invitrogen, Life Technologies, Carlsbad, CA, USA) under reducing conditions, as per manufacturer's instructions. After protein separation, gels were stained with colloidal Coomassie Blue (SimplyBlue™ SafeStain, Invitrogen) then destained in distilled water. Mass spectrometry analysis was carried out at the Moredun Research Institute's Proteomics Facility as described previously (Moredun, 2011; Smith *et al.* 2009). Mascot generic (mgf) files were generated from the resultant data and submitted to a local database server using the MASCOT search engine for protein database searching against NCBI non-redundant database (<http://www.ncbi.nlm.nih.gov>) with mammals as a taxonomical search parameter. The fixed and variable modifications used in the searches were carbamidomethyl (C) and oxidation (M) respectively. Mass tolerance values for MS and MS/MS were set at 1.5 Da and 0.5 Da, respectively. Matches achieving a significant molecular weight search (MOWSE) score were considered significant only if 2 or more peptides were matched for each protein, and each was observed to contain an unbroken 'b' or 'y' ion series of a minimum of 4 amino acid residues.

RESULTS

Ex-sheathing larvae

The percentage of ex-sheathed larvae at each time-point sampled during the ex-sheathing process and during the exposure to the abomasal environment samples are shown in Fig. 1. After incubation for 2 h in EBSS ex-sheathing buffer, 15% of the larvae had ex-sheathed. After 3 h, 49% had ex-sheathed and after 4 h 67% had ex-sheathed. At each of these time-points no larvae had died (data not shown). After incubation for 4 h in ex-sheathing buffer the larvae were transferred to incubation in either the immune or naïve abomasal environment samples. The numbers of larvae that had ex-sheathed after 2 h and 4 h in this

Table 1. Overview of bioinformatic analysis

(a, Isotigs which share 1 or more 454 reads are grouped by the Newbler assembler into 'Isogroups', so that the Isotigs within 1 Isogroup could represent the alternative isoforms from the same gene.)

	Immune exposed	Naïve exposed
Raw data and assembly information		
Number of reads	82 096	82 267
Number assembling into Isotigs	59 240 (72%)	63 825 (78%)
Number of Isotigs	5525	5395
Number of Isogroups ^a	4651	4617
Homology searches(Using Isotig consensus sequences)		
With homology to sequences in NCBI non-redundant database	3736 (68%)	3550 (66%)
With predicted gene ontology (GO) terms	2651 (48%)	2560 (47%)
Returning InterProScan results	2733 (49%)	3078 (57%)
Returning enzyme match	848 (15%)	724 (13%)
Biological pathways predicted (KEGG)	104	98
Total number of Clusters (combined dataset)	6969	

environment are represented by the 6-n and 8-n bars (incubated in naïve environment sample) and the 6-i and 8-i bars (incubated in immune environment sample) in Fig. 1. After incubation for 8 h, more than 99% of the larvae were ex-sheathed and alive.

Bioinformatic analysis

An overview of the sequencing data, assembly and initial bioinformatic analysis is shown in Table 1. The number of Isotigs in each dataset with Gene Ontology annotations, InterProScan results and enzyme matches, and the total number of Clusters obtained after combining the 2 sets of Isotigs, are also shown.

Gene ontology

The level 2 Gene Ontology terms associated with the Isotigs from each sample are shown in Table 2. Table 3 shows the 'biological process' and 'cellular component' GO terms that had a significantly higher mean proportion of Isotigs in the immune exposed group than the naïve exposed group. For biological processes these included 'carbon utilization', 'response to stimulus' and 'developmental process' as exemplified by CRE-ALDO-2 protein (fructose-bisphosphate aldolase 2, 44 Isotigs), heat shock protein 16-2 (68 Isotigs) and heat shock protein 16-2 (68 Isotigs)/CRE-GEI-7 protein (isocitrate lyase-like protein, 65 Isotigs) respectively (all $P_F < 0.001$). Within 'cellular component', mean proportion of Isotig for 'membrane-enclosed lumen' and 'macromolecular complex' were statistically significantly higher in the immune-exposed group compared to the naïve-exposed group ($P_F \leq 0.001$). There was no evidence that the number of any level 2 'molecular function' annotations were statistically different between the exposed and naïve group.

KEGG biological pathways

KEGG biological pathway annotation of the 2 sets of Isotigs was carried out within the Blast2GO software suite and pathways showing statistically significant differences in the mean proportion of Isotigs between the two groups are shown in Table 4. Pathways showing statistically significantly higher mean proportion of Isotigs in the larvae exposed to the immune environment ($P_F \leq 0.001$) were 'carbohydrate metabolism', 'biosynthesis of alkaloids', 'biosynthesis of phenylpropanoids, plant hormones, terpenoids and steroids', 'biosynthesis of secondary metabolites' and 'metabolic pathways'. Immune exposed larvae also showed a statistically significantly higher number of Isotigs for metabolic pathways ($P_F = 0.039$).

Most abundant transcripts

Clustering of combined sets of 5525 Isotigs from the immune exposed larval sample and the 5395 Isotigs from the naïve-exposed sample using UCLUST software resulted in 6969 Clusters. The 10 most abundant sequences for each sample, determined by the number of unique reads contributing to the Cluster, are shown in Table 5. In the sample that had been prepared from larvae exposed to the immune abomasal environment, the most abundant sequences were a homologue of a hypothetical protein homologue from *Brugia malayi*, an unidentified protein that had no hits in the NCBI non-redundant database, and a senescence-associated protein also from *B. malayi*. The most abundant sequences in the naïve-exposed larval sample were the same hypothetical protein homologue from *B. malayi* identified in the immune-exposed larvae, Cytochrome b and NADH dehydrogenase subunit 5.

Table 2. Parental (level 2) gene ontology (GO) terms associated with the assembled Isotigs from each sample

(GO categories were assigned within the Blast2GO software according to hits from the NCBI non-redundant database, and also according to InterPro domains with homology to functionally annotated genes.)

	Number of Isotigs (%)	
	Immune exposed	Naïve exposed
<i>Biological Process (GO:0008150)</i>	2164 (39.2)	2088 (38.7)
cellular process (GO:0009987)	1524 (27.6)	1320 (24.5)
metabolic process (GO:0008152)	1246 (22.6)	1220 (22.6)
multicellular organismal process (GO:0032501)	1299 (23.5)	1070 (19.8)
developmental process (GO:0032502)	1244 (22.5)	996 (18.5)
biological regulation (GO:0065007)	1026 (18.6)	838 (15.5)
growth (GO:0040007)	860 (15.6)	658 (12.2)
reproduction (GO:0000003)	723 (13.1)	627 (11.6)
localization (GO:0051179)	599 (10.8)	583 (10.8)
locomotion (GO:0040011)	440 (8.0)	375 (7.0)
cellular component organization (GO:0016043)	365 (6.6)	360 (6.7)
response to stimulus (GO:0050896)	416 (7.5)	255 (4.7)
signaling (GO:0023052)	289 (5.2)	250 (4.6)
cellular component biogenesis (GO:0044085)	114 (2.1)	74 (1.4)
death (GO:0016265)	39 (0.7)	31 (0.6)
biological adhesion (GO:0022610)	13 (0.2)	20 (0.4)
multi-organism process (GO:0051704)	10 (0.2)	17 (0.3)
rhythmic process (GO:0048511)	4 (0.1)	4 (0.1)
cell proliferation (GO:0008283)	5 (0.1)	4 (0.1)
cell wall organization or biogenesis (GO:0071554)	5 (0.1)	3 (0.1)
nitrogen utilization (GO:0019740)	1 (0.0)	2 (0.0)
carbon utilization (GO:0015976)	58 (1.0)	2 (0.0)
pigmentation (GO:0043473)	0 (0.0)	1 (0.0)
immune system process (GO:0002376)	3 (0.1)	1 (0.0)
viral reproduction (GO:0016032)	0 (0.0)	1 (0.0)
cell killing (GO:0001906)	1 (0.0)	0 (0.0)
<i>Cellular Component (GO:0005575)</i>	1350 (24.4)	1297 (24.0)
cell (GO:0005623)	1289 (23.3)	1210 (22.4)
organelle (GO:0043226)	604 (10.9)	598 (11.1)
macromolecular complex (GO:0032991)	397 (7.2)	281 (5.2)
extracellular region (GO:0005576)	97 (1.8)	99 (1.8)
membrane-enclosed lumen (GO:0031974)	91 (1.6)	31 (0.6)
synapse (GO:0045202)	29 (0.5)	26 (0.5)
<i>Molecular function (GO:0003674)</i>	2128 (38.5)	2114 (39.2)
binding (GO:0005488)	1534 (27.8)	1523 (28.2)
catalytic activity (GO:0003824)	1225 (22.2)	1139 (21.1)
transporter activity (GO:0005215)	194 (3.5)	155 (2.9)
structural molecule activity (GO:0005198)	99 (1.8)	118 (2.2)
molecular transducer activity (GO:0060089)	69 (1.2)	70 (1.3)
electron carrier activity (GO:0009055)	47 (0.9)	68 (1.3)
enzyme regulator activity (GO:0030234)	51 (0.9)	64 (1.2)
transcription regulator activity (GO:0030528)	64 (1.2)	56 (1.0)
antioxidant activity (GO:0016209)	15 (0.3)	14 (0.3)
metallochaperone activity (GO:0016530)	1 (0.0)	1 (0.0)
channel regulator activity (GO:0016247)	2 (0.0)	0 (0.0)

Differences in sequence abundance between the larval samples

Individual sequencing reads contributing to each Cluster were identified, and parsed to output the number of unique reads per cluster originating from each sample. Analysis of these Cluster read counts resulted in 18 Clusters showing statistical significance at levels consistent with a false discovery rate of less than 5% ($P_F < 0.05$) for differences in mean

proportions of reads between the naïve and immune groups under the conservative model, while a further 77 Clusters were of indeterminate status, being statistically significant with $P_F < 0.05$ under the liberal model only. The 18 Clusters with $P_F \leq 0.05$ under the conservative model and the identity of the representative sequence of each (determined by Blastx homology searching to proteins in the NCBI non-redundant database) are shown in Table 6. A total of 11 Clusters had statistically significantly

Table 3. GO terms with significantly more abundant representation within the immune exposed larval

	Number of Isotigs (%)		<i>P</i> (FDR adjusted)
	Immune exposed	Naïve exposed	
Biological process			
carbon utilization	58 (1.0)	2 (0.0)	<0.001
response to stimulus	416 (7.5)	255 (4.7)	<0.001
developmental process	1244 (22.5)	996 (18.4)	<0.001
growth	860 (15.5)	658 (12.2)	<0.001
multicellular organismal process	1299 (23.5)	1070 (19.8)	<0.001
biological regulation	1026 (18.5)	838 (15.5)	<0.001
cellular process	1524 (27.5)	1320 (24.4)	<0.001
cellular component biogenesis	114 (2.1)	74 (1.4)	0.020
Cellular component			
membrane-enclosed lumen	91 (1.6)	31 (0.6)	<0.001
macromolecular complex	397 (7.2)	281 (5.2)	<0.001

Table 4. KEGG annotations showing significant differences in abundance between the immune and naïve exposed larvae

Pathway category	Number of sequences (%)		<i>P</i> (FDR adjusted)
	Immune exposed	Naïve exposed	
Carbohydrate metabolism	481 (8.7)	216 (4.0)	<0.001
Biosynthesis of alkaloids	349 (6.3)	155 (2.9)	<0.001
Biosynthesis of phenylpropanoids, plant hormones, terpenoids and steroids	273 (4.9)	120 (2.2)	<0.001
Biosynthesis of secondary metabolites	151 (2.7)	71 (1.3)	<0.001
Metabolic pathways	367 (6.6)	293 (5.4)	0.039

higher mean proportion of reads in the immune-exposed larvae than naïve larvae ($P_F \leq 0.001$). These included Cluster 295 (CBR-PGAL-1 protein – a peptidylglycine alpha-amidating mono-oxygenase homologue), Cluster 520 (heat shock protein 16-2), Cluster 273 (NADH dehydrogenase subunit 4) and Cluster 2240 (CRE-IDA-1 protein, a tyrosine phosphatase-like receptor protein). A total of 7 Clusters had a statistically significantly greater mean proportion of reads in the naïve larval sample ($P_F \leq 0.001$) which included Cluster 334 (cytochrome b), Cluster 108 (EGG Laying defective family member (EGL-21) and Cluster 538 (NADH dehydrogenase subunit 5). Four of the 11 Clusters with $P_F \leq 0.05$ and a higher mean proportion of reads originating from the immune exposed larvae, and 3 of the 7 Clusters with $P_F \leq 0.05$ and a higher mean proportion of reads originating from the naïve-exposed larvae, were also amongst the 10 Clusters with the highest read counts for their respective originating samples. Furthermore, 8 of the 11 Clusters with $P_F \leq 0.05$ for the immune-exposed larvae appeared to be unique to that dataset, with no representative reads from the naïve-exposed larval group.

In the set of indeterminate Clusters with $P_F \leq 0.05$ only under the liberal model (Table 7), the immune-exposed group had a statistically significantly higher

mean proportion of reads for sequences (with top Blastx hits) of CRE-EFT-2 (translation elongation factor 2), *Brachionus plicatilis* LEA-like protein 1 (late embryo abundant), the *B. malayi* senescence-associated protein and *T. circumcincta* GTP-Cyclohydrolase. The indeterminate clusters that showed a statistically significantly higher mean proportion of read counts in naïve-exposed larvae relative to immune-exposed larvae returned sequences (top Blastx hits) of *Caenorhabditis briggsae* UNC-54 protein (myosin heavy chain), *C. briggsae* putative acyltransferase, *Oesophagostomum dentatum* FMRFamide-like propeptide (*flp-14*), *Caenorhabditis elegans* UBC-18 homologue (ubiquitin-conjugating enzyme) and *Caenorhabditis remanei* EPN-1 protein (nucleoside transporter protein).

Proteomics results

Analysis of the proteins present in the naïve and immune abomasal environment samples returned 702 and 695 matches respectively in the NCBI nr database. After *in silico* subtraction and manual curation to remove matches with the same identity, this was reduced to 67 protein matches uniquely detected in the naïve abomasal environment, and

Table 5. Top 10 most abundant sequences in each dataset, by number of reads from sample present in cluster

(a, Cluster appears in top 10 most abundant in both samples; -, no hits in NCBI database. b, Blastx search using representative sequence as query.)

Cluster	Number of reads	Length of rep. seq. (bp)	Best alignment in NCBI non-redundant database ^b			
			Species	Identity	Accession no.	e-value
Immune exposed larvae						
Cluster 15 ^a	3479	2561	<i>Brugia malayi</i>	Hypothetical protein Bm_11025	EDP37487.1	6E-23
Cluster 611	1739	947	—	—	—	—
Cluster 2 ^a	1308	4021	<i>Brugia malayi</i>	Senescence-associated protein	EDP31077.1	5E-57
Cluster 6	957	3036	<i>Caenorhabditis remanei</i>	CRE-EFT-2 protein	EFP00955.1	0.0
Cluster 95 ^a	813	1715	<i>Caenorhabditis elegans</i>	C-term-of-AEX-Binding protein family member (cab- 1)	AAG17881.1	2E-62
Cluster 968 ^a	790	781	<i>Teladorsagia circumcincta</i>	Cytochrome c oxidase subunit ii	ACX85202.1	1E-110
Cluster 290 ^a	708	1235	<i>Caenorhabditis elegans</i>	hypothetical protein Y71G12B.4	AAK73930.1	8E-121
Cluster 26	666	2328	<i>Haemonchus contortus</i>	Phosphoenolpyruvate carboxykinase	AAA29180.1	0.0
Cluster 137 ^a	664	1582	<i>T. circumcincta</i>	NADH dehydrogenase subunit 5	ACX85204.1	0.0
Cluster 295	664	1228	<i>Caenorhabditis briggsae</i>	CBR-PGAL-1 protein	CAP37494.1	2E-101
Naïve exposed larvae						
Cluster 15 ^a	2653	2561	<i>Brugia malayi</i>	Hypothetical protein Bm_11025	EDP37487.1	6E-23
Cluster 334	1861	1173	<i>Teladorsagia circumcincta</i>	Cytochrome b	ACX85210.1	3E-143
Cluster 137 ^a	1279	1582	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit 5	ACX85204.1	0.0
Cluster 135	1276	1584	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit 1	ACX85207.1	6E-112
Cluster 2 ^a	1078	4021	<i>Brugia malayi</i>	Senescence-associated protein	EDP31077.1	5E-57
Cluster 968 ^a	946	781	<i>Teladorsagia circumcincta</i>	Cytochrome c oxidase subunit ii	ACX85202.1	1E-110
Cluster 475	909	1047	<i>Caenorhabditis elegans</i>	C-type LECTin family member (clec-1)	AAB37085.1	1E-50
Cluster 95 ^a	749	1715	<i>Caenorhabditis elegans</i>	C-term-of-AEX-Binding protein family member (cab- 1)	AAG17881.1	2E-62
Cluster 1225	721	704	—	—	—	—
Cluster 290 ^a	704	1235	<i>Caenorhabditis elegans</i>	hypothetical protein Y71G12B.4	AAK73930.1	8E-121

67 protein matches uniquely detected in the immune abomasal environment sample (Table 8). Proteins identified uniquely in the immune sample included clathrin, intelectin-2, galectin-15, IgE and gelsolin-b.

DISCUSSION

In this study, we have presented data comparing the transcriptome of exsheathed infective *Teladorsagia circumcincta* 3rd larval stage parasites upon exposure to either a naïve or immune host environment, and also carried out qualitative proteomic analysis on the host environment samples. These data showed changes in transcript levels between the 2 larval populations, particularly for several genes with possible roles in sensory capabilities and response to the differing environments, in this case possibly reflecting early responses to the host immune response. The proteome profiles of the naïve and immune abomasal environments confirmed that the larvae exposed to the immune environment encountered relevant immune effectors.

A conservative statistical model was used, due to the lack of experimental repeats, to determine statistically significant differences in transcript

abundance between the transcriptomes of the 2 larval samples. Eighteen transcripts were identified although, as a conservative statistical model was employed, the true number of transcripts with significantly differing abundance may be higher than this. O'Meara *et al.* (2010) also observed that changes in expression occurred in only a relatively small proportion of *Strongyloides ratti* transcripts in response to host immune status.

Analysis of the GOs associated with the entire transcriptome from each sample (i.e. not restricted to only those transcripts with higher mean abundance in the immune-exposed larvae) showed that in the immune-environment exposed larval sample the most statistically significantly increased annotations were 'carbon utilization', 'response to stimulus' and 'developmental process'. The increased number of transcripts with these annotations is likely to reflect the response of larvae encountering a different environment from those exposed to a naïve host environment.

One of the transcripts with the greatest increase in abundance in larvae exposed to the immune host environment was a *C. briggsae* peptidyl-glycine alpha-amidating monooxygenase protein homologue (CBR-PGAL-1 protein). The peptidyl-glycine

Table 6. Identities of Clusters with statistically significant differences in mean read count abundance between immune-exposed and naïve-exposed groups under the conservative model

(a, cluster also amongst the top 10 most abundant for that sample. b, Blastx search using representative sequence as query. -, no hits in NCBI nr database.)

	Read counts			Best alignment in NCBI non-redundant database ^b			
Cluster	Immune exposed	Naïve exposed	<i>P</i> (FDR adjusted)	Species	Identity	Accession	<i>e</i> -value
Clusters containing significantly more reads originating from immune exposed larvae							
Cluster 295 ^a	664	0	<0.001	<i>Caenorhabditis briggsae</i>	CBR-PGAL-1 protein	CAP37494.1	1.9E-101
Cluster 520	446	0	<0.001	<i>Caenorhabditis elegans</i>	Heat shock protein 16-2	AAA28071.1	3.6E-21
Cluster 273	417	0	<0.001	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit 4	ACX85212.1	3.3E-96
Cluster 2240	147	0	<0.001	<i>Caenorhabditis remanei</i>	CRE-IDA-1 protein	EFO86750.1	1.3E-15
Cluster 242	298	44	<0.001	<i>Caenorhabditis remanei</i>	CRE-ALDO-2 protein	EFO86555.1	8.8E-163
Cluster 26 ^a	666	293	<0.001	<i>Haemonchus contortus</i>	Phosphoenolpyruvate carboxykinase	AAA29180.1	0.0
Cluster 15 ^a	3479	2653	<0.001	<i>Brugia malayi</i>	Hypothetical protein Bm_11025	EDP37487.1	6.2E-23
Cluster 611 ^a	1739	0	0.010	—	—	—	—
Cluster 3523	48	0	0.017	—	—	—	—
Cluster 994	45	0	0.029	—	— (IPS matches: COIL, PTHR23241, seg.)	—	—
Cluster 6794	43	0	0.038	—	—	—	—
Clusters containing significantly more reads originating from naive exposed larvae							
Cluster 334 ^a	158	1861	<0.001	<i>Teladorsagia circumcincta</i>	Cytochrome b	ACX85210.1	3.5E-143
Cluster 108	32	449	<0.001	<i>Caenorhabditis elegans</i>	EGg Laying defective family member (egl-21)	CAB02881.1	0.0
Cluster 538	0	532	<0.001	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit 5	ACX85204.1	4.1E-62
Cluster 1091	338	686	<0.001	<i>Teladorsagia circumcincta</i>	cytochrome c oxidase subunit 1	ACX85201.1	8.8E-110
Cluster 475 ^a	2	909	0.003	<i>Caenorhabditis elegans</i>	C-type LECTin family member (clec-1)	AAB37085.1	1.3E-50
Cluster 135 ^a	0	1276	0.010	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit 1	ACX85207.1	7.6E-112
Cluster 2749	11	116	0.038	—	— (IPS match: TMHMM)	—	—

alpha-amidating monooxygenase gene encodes 1 polypeptide with 2 enzymatic domains, responsible for the copper-dependent alpha-amidation of glycine-extended biopeptides often necessary for full activity of the peptides (Eipper *et al.* 1993; Prigge *et al.* 2000). Neuropeptides are commonly modified at the N- or C-terminus to protect them from degradation and/or to achieve full biological activity. Amidated peptides have widespread functions in the nervous system, and in development and regulation: in *C. elegans* over 18 genes encode more than 53 Phe-Met-Arg-Phe-NH₂ (FMRFamide) related peptides, yet disruption of a single precursor gene, and the resultant varying levels of the peptide, leads to a profound variation in behaviour (Nelson *et al.* 1998). In *Schistosoma mansoni* the release of immunosuppressive neuropeptides has been associated with deactivation of previously activated host lymphocytes, an example of helminth immunomodulation via molecular mimicry, a phenomenon whereby parasites mimic endogenous host proteins in order to interfere with the host response (Duvaux-Miret *et al.* 1992). The greater abundance of the CBR-PGAL-1 homologue transcript in larvae exposed to the immune host environment as opposed to those exposed to the naïve environment raises the possibility that this transcript is involved in neuropeptide processing in response to the host immune factors present, and may play a role in either evasion and/or modulation of the host immune response, or may be involved in changes in developmental regulation related to environmental cues.

The second most abundant transcript in the larvae exposed to the immune abomasal environment (named 'Cluster 611') was exclusive to that dataset and database searches (Wormbase, 2011) indicated significant homology to *C. elegans* mitochondrial DNA.

Another transcript present in higher abundance in the immune-exposed larvae, which may be indicative of a response to encountering the immune host environment, was an IDA-1 homologue (in *C. remanei*), which is a tyrosine phosphatase-like receptor protein. The *C. elegans* homologue of CRE-IDA-1 is a membrane-spanning protein associated with the granules of neural- and endocrine-specific cells, involved in the release of neuropeptides from dense core vesicles, with parallels to insulin signalling in mammals (Zahn *et al.* 2001; Cai *et al.* 2004). An insulin-like pathway is known to regulate dauer formation in *C. elegans* following environmental cues (Thomas *et al.* 1993; Gottlieb and Ruvkun, 1994; Riddle and Albert, 1997; Hu, 2007). The *T. circumcincta* larvae described herein are entering an environment known to cause immune-mediated inhibition of larval development (McKellar, 1993; Halliday *et al.* 2007; Smith, 2007) therefore the possibility also exists of the IDA-1 homologue protein being involved in a signalling pathway

involved in arrested development in response to environmental cues.

Other transcripts with a statistically significantly higher mean abundance in the immune-exposed larvae included the stress-induced heat shock protein 16-2 (HSP 16-2; (Ding and Candido, 2000)). The host immune system will be producing oxidative free radicals to kill pathogens, and HSP 16-2 has been found to protect cells from death due to such oxidative stress (Bellyei *et al.* 2007; Hartwig *et al.* 2009). A *C. remanei* ALDO-2 (fructose biphosphase aldolase) homologue and an *H. contortus* phosphoenolpyruvate carboxykinase homologue also displayed greater abundance in the immune-exposed larvae, indicating increased carbon utilization.

Amongst the list of indeterminate Clusters with $P_F \leq 0.05$ only under the liberal model, sequences showing higher abundance of reads in immune-exposed larvae included a Late Embryo Abundant protein homologue (LEA-1) and *T. circumcincta* GTP-Cyclohydrolase. The *lea-1* gene encodes a protein that is involved in the stress-response mechanism in *C. elegans*, particularly desiccation, osmotic and heat stress (Gal *et al.* 2004). GTP-cyclohydrolase (GTP-CH) has been identified as one of the most abundant transcripts in the exsheathed L3 *T. circumcincta* transcriptome when compared to the 4th larval stage (Nisbet *et al.* 2008), and the *C. elegans cat-4* gene which encodes GTP-cyclohydrolase shows increased transcription in *C. elegans* dauer larvae (Jeong *et al.* 2009). GTP-cyclohydrolase has a proposed role in environmental protection in *T. circumcincta* larval stages with transcript levels observed to fall in *T. circumcincta* upon transition to the parasitic lifestyle (Baker *et al.* 2011), thus raising the possibility that maintenance of higher levels of transcription in larvae exposed to the immune environment in these experiments may reflect a requirement for ongoing environmental protection.

Within the sequences which had a statistically significantly higher mean proportion of reads in the larvae exposed to the naïve host environment were transcripts for proteins involved in the respiratory electron transport chain; a *C. elegans* EGg Laying defective family member (*egl-21*) homologue (a carboxypeptidase which facilitates acetylcholine release at neuromuscular junctions (Jacob and Kaplan, 2003)); and a *C. elegans* C-type LECTin family member (*clec-1*) homologue with associated biological process Gene Ontologies of body morphogenesis, growth, hermaphrodite genitalia development, locomotion, morphogenesis of an epithelium, nematode larval development and positive regulation of multicellular organism growth. These transcripts which are more abundant in the larvae exposed to the naïve host environment, are consistent with parasites growing and developing as would be predicted given current knowledge of the *T. circumcincta* life cycle.

Table 7. Identities of indeterminate Clusters with statistically significant differences in mean read count abundance between immune-exposed and naïve-exposed groups under the liberal model

(a, Blastx search using representative sequence as query; -, no hits in NCBI nr database.)

Cluster	Read counts		<i>P</i> (FDR adjusted)	Top hit protein in NCBI nr database*				
	Naïve exposed	Immune exposed		Species	Identity	Accession	<i>E</i> -value	Rank (/77)
Clusters containing more reads originating from immune exposed larvae								
Cluster 6	696	957	<0.001	<i>Caenorhabditis remanei</i>	CRE-EFT-2 protein	XP_003105057	0	2
Cluster 6854	0	27	<0.001	—	—	—	—	5
Cluster 2363	0	25	<0.001	—	—	—	—	6
Cluster 289	2	91	<0.001	<i>Brachionus plicatilis</i>	LEA-like protein 1	ADE05593	3.94E-06	10
Cluster 1929	5	52	<0.001	—	—	—	—	14
Cluster 1805	0	20	<0.001	<i>Caenorhabditis briggsae</i>	hypothetical protein	XP_002640908	2.05E-72	15
Cluster 2	1078	1308	<0.001	<i>Tetrahymena thermophila</i>	senescence-associated protein	XP_001028745	1.66E-58	17
Cluster 614	292	409	0.001	<i>Teladorsagia circumcincta</i>	GTP-Cyclohydrolase	CBX89810.1	5.00E-111	20
Cluster 4966	0	17	0.001	—	—	—	—	21
Cluster 2994	0	16	0.002	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_12628	XP_003107924.1	3.85E-38	25
Cluster 616	5	35	0.006	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_22750	CAP39270.2	8.76E-71	32
Cluster 71	3	34	0.007	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_02279	XP_003117095.1	1.58E-161	33
Cluster 461	0	14	0.007	<i>Caenorhabditis elegans</i>	Guanylyl CYclase family member	NP_497026.2	3.72E-68	34
Cluster 1117	0	14	0.007	—	—	—	—	35
Cluster 1658	0	14	0.007	—	—	—	—	36
Cluster 5579	0	14	0.007	<i>Angiostrongylus cantonensis</i>	putative Dynein Heavy Chain	CAR63664.1	3.95E-30	37
Cluster 72	63	115	0.013	<i>Caenorhabditis briggsae</i>	CBR-BRP-1 protein	XP_002642206.1	2.37E-16	40
Cluster 4063	0	13	0.013	—	—	—	—	41
Cluster 5627	0	13	0.013	<i>Caenorhabditis elegans</i>	hypothetical protein Y57E12AL.1	NP_741562	1.27E-36	42
Cluster 6795	0	13	0.013	—	—	—	—	43
Cluster 1593	1	46	0.016	<i>Brugia malayi</i>	hypothetical protein Bm1_25640	XP_001896587.1	1.95E-46	45
Cluster 458	2	31	0.018	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_25163	XP_003113223	1.60E-51	46
Cluster 564	0	12	0.023	<i>Caenorhabditis elegans</i>	hypothetical protein T12A2.2	NP_498362.1	8.19E-118	48
Cluster 615	0	12	0.023	<i>Brugia malayi</i>	UDP-glucoronosyl and UDP-glucosyl	XP_001894161.1	2.03E-59	49
Cluster 878	0	12	0.023	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_25063	XP_003113058.1	1.44E-12	50
Cluster 1436	0	12	0.023	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_27997	CAS00988.1	6.71E-48	51
Cluster 2627	0	12	0.023	—	—	—	—	52
Cluster 813	94	150	0.028	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit II	YP_003434086.1	3.33E-55	60
Cluster 23	16	45	0.031	<i>Caenorhabditis elegans</i>	hypothetical protein C34D4.14	NP_501120.1	0	61
Cluster 146	0	11	0.036	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_24093	XP_003099813.1	1.19E-82	63
Cluster 197	0	11	0.036	<i>Caenorhabditis elegans</i>	Dynein Heavy Chain family member	NP_491363.1	7.73E-153	64
Cluster 430	0	11	0.036	<i>Caenorhabditis briggsae</i>	hypothetical protein CBG21629	XP_002632697.1	5.15E-113	65
Cluster 474	0	11	0.036	<i>Caenorhabditis elegans</i>	UNCoordinated family member	NP_001023017.1	8.38E-50	66
Cluster 900	0	11	0.036	<i>Caenorhabditis elegans</i>	hypothetical protein F15C11.2	NP_740883.1	6.34E-13	67
Cluster 1040	0	11	0.036	<i>Caenorhabditis elegans</i>	hypothetical protein E01G6.3	NP_510042.2	8.75E-25	68
Cluster 1192	0	11	0.036	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_00943	CAP22305.2	5.93E-67	69
Cluster 1827	0	11	0.036	<i>Caenorhabditis briggsae</i>	hypothetical protein CBG12633	XP_002640134.1	6.04E-54	70

Cluster 2983	0	11	0.036	<i>Steinernema carpocapsae</i>	saposin precursor	ADG63466.1	7.70E-42	71
Cluster 3175	0	11	0.036	<i>Loa loa</i>	hypothetical protein LOAG_04581	EFO23902.1	6.64E-62	72
Cluster 847	4	25	0.047	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_15976	XP_003106503.1	1.95E-62	77
Clusters containing more reads originating from naïve exposed larvae								
Cluster 6	696	957	<0.001	<i>Caenorhabditis remanei</i>	CRE-EFT-2 protein	XP_003105057	0	2
Cluster 6854	0	27	<0.001	—	—	—	—	5
Cluster 2363	0	25	<0.001	—	—	—	—	6
Cluster 289	2	91	<0.001	<i>Brachionus plicatilis</i>	LEA-like protein 1	ADE05593	3.94E-06	10
Cluster 1929	5	52	<0.001	—	—	—	—	14
Cluster 1805	0	20	<0.001	<i>Caenorhabditis briggsae</i>	hypothetical protein CBG00466	XP_002640908	2.05E-72	15
Cluster 2	1078	1308	<0.001	<i>Tetrahymena thermophila</i>	senescence-associated protein	XP_001028745	1.66E-58	17
Cluster 614	292	409	0.001	<i>Teladorsagia circumcincta</i>	GTP-Cyclohydrolase	CBX89810.1	5.00E-111	20
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Cluster 2994	0	16	0.002	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_12628	XP_003107924.1	3.85E-38	25
Cluster 616	5	35	0.006	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_22750	CAP39270.2	8.76E-71	32
Cluster 71	3	34	0.007	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_02279	XP_003117095.1	1.58E-161	33
Cluster 461	0	14	0.007	<i>Caenorhabditis elegans</i>	Guanylyl CYclase family member	NP_497026.2	3.72E-68	34
Cluster 1117	0	14	0.007	—	—	—	—	35
Cluster 1658	0	14	0.007	—	—	—	—	36
Cluster 5579	0	14	0.007	<i>Angiostrongylus cantonensis</i>	putative Dynein Heavy Chain	CAR63664.1	3.95E-30	37
Cluster 72	63	115	0.013	<i>Caenorhabditis briggsae</i>	CBR-BRP-1 protein	XP_002642206.1	2.37E-16	40
Cluster 4063	0	13	0.013	—	—	—	—	41
Cluster 5627	0	13	0.013	<i>Caenorhabditis elegans</i>	hypothetical protein Y57E12AL.1	NP_741562	1.27E-36	42
Cluster 6795	0	13	0.013	—	—	—	—	43
Cluster 1593	1	46	0.016	<i>Brugia malayi</i>	hypothetical protein Bm1_25640	XP_001896587.1	1.95E-46	45
Cluster 458	2	31	0.018	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_25163	XP_003113223	1.60E-51	46
Cluster 564	0	12	0.023	<i>Caenorhabditis elegans</i>	hypothetical protein T12A2.2	NP_498362.1	8.19E-118	48
Cluster 615	0	12	0.023	<i>Brugia malayi</i>	UDP-glucuronosyl and UDP-glucosyl	XP_001894161.1	2.03E-59	49
Cluster 878	0	12	0.023	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_25063	XP_003113058.1	1.44E-12	50
Cluster 1436	0	12	0.023	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_27997	CAS00988.1	6.71E-48	51
Cluster 2627	0	12	0.023	—	—	—	—	52
Cluster 813	94	150	0.028	<i>Teladorsagia circumcincta</i>	NADH dehydrogenase subunit II	YP_003434086.1	3.33E-55	60
Cluster 23	16	45	0.031	<i>Caenorhabditis elegans</i>	hypothetical protein C34D4.14	NP_501120.1	0	61
Cluster 146	0	11	0.036	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_24093	XP_003099813.1	1.19E-82	63
Cluster 197	0	11	0.036	<i>Caenorhabditis elegans</i>	Dynein Heavy Chain family member	NP_491363.1	7.73E-153	64
Cluster 430	0	11	0.036	<i>Caenorhabditis briggsae</i>	hypothetical protein CBG21629	XP_002632697.1	5.15E-113	65
Cluster 474	0	11	0.036	<i>Caenorhabditis elegans</i>	UNCoordinated family member	NP_001023017.1	8.38E-50	66
Cluster 900	0	11	0.036	<i>Caenorhabditis elegans</i>	hypothetical protein F15C11.2	NP_740883.1	6.34E-13	67
Cluster 1040	0	11	0.036	<i>Caenorhabditis elegans</i>	hypothetical protein E01G6.3	NP_510042.2	8.75E-25	68
Cluster 1192	0	11	0.036	<i>Caenorhabditis briggsae AF16</i>	hypothetical protein CBG_00943	CAP22305.2	5.93E-67	69
Cluster 1827	0	11	0.036	<i>Caenorhabditis briggsae</i>	hypothetical protein CBG12633	XP_002640134.1	6.04E-54	70
Cluster 2983	0	11	0.036	<i>Steinernema carpocapsae</i>	saposin precursor	ADG63466.1	7.70E-42	71
Cluster 3175	0	11	0.036	<i>Loa loa</i>	hypothetical protein LOAG_04581	EFO23902.1	6.64E-62	72
Cluster 847	4	25	0.047	<i>Caenorhabditis remanei</i>	hypothetical protein CRE_15976	XP_003106503.1	1.95E-62	77

* Representative sequence from cluster used as query for Blastx search of NCBI non-redundant database.

— no hits in NCBI nr database.

Table 8. Proteins exclusively present in either immune or naïve abomasal environment samples, identified by LC-MS/MS

(A match was considered significant when the number of non-redundant peptides with 4 or more consecutive *b* or *y* ions was greater than or equal to 2. a, For each sequence that provided a significant match, only the gel slice containing the highest MOWSE value for that protein is indicated. b, Sequence coverage is expressed as the number of amino acids spanned by the assigned peptide divided by the sequence length. c, The total number of non-redundant peptides assigned to the protein is shown. When calculating this number, multiple matches to peptides with the same primary sequence counted as 1, even if they represented different charge states or modification states. d, The Accession number and identity of the highest hit identified in the NCBI non-redundant database is shown.)

Gel slice ^a	MOWSE score	Sequence coverage (%) ^b	Total no. peptides ^c	GenInfo Identifier ^d	Species	Identity
Immune abomasal environment						
2	152	2	9	gi 40849908	<i>Mus musculus</i>	plectin 1
4	286	20	23	gi 114669627	<i>Pan troglodytes</i>	PREDICTED: clathrin heavy chain 1 isoform 7
5	396	29	20	gi 297471223	<i>Bos taurus</i>	PREDICTED: ceruloplasmin (ferroxidase)
5	60	7	5	gi 73990367	<i>Canis familiaris</i>	PREDICTED: similar to Ceruloplasmin precursor (Ferroxidase) isoform 1
5	93	3	3	gi 76677897	<i>Bos taurus</i>	complement factor H precursor
6	88	6	3	gi 110331845	<i>Bos taurus</i>	inter-alpha (globulin) inhibitor H3
6	66	2	2	gi 149056486	<i>Rattus norvegicus</i>	rCG54015
7	147	13	8	gi 114583316	<i>Pan troglodytes</i>	PREDICTED: villin 1 isoform 2
7	242	29	16	gi 296190199	<i>Callithrix jacchus</i>	PREDICTED: transitional endoplasmic reticulum ATPase-like
7	112	7	6	gi 297462670	<i>Bos taurus</i>	PREDICTED: primary amine oxidase, liver isozyme-like
7	107	6	3	gi 114626425	<i>Pan troglodytes</i>	PREDICTED: gelsolin isoform 3
7	83	3	2	gi 297290486	<i>Macaca mulatta</i>	PREDICTED: complement factor B isoform 1
8	49	11	4	gi 165926	<i>Ovis aries</i>	immunoglobulin epsilon-chain
8	66	6	2	gi 191765	<i>Mus musculus</i>	alpha-fetoprotein
8	77	5	4	gi 301777396	<i>Ailuropoda melanoleuca</i>	PREDICTED: inhibitor of carbonic anhydrase-like
8	226	18	8	gi 149038929	<i>Rattus norvegicus</i>	gelsolin, isoform CRA_b
8	241	22	10	gi 296212544	<i>Callithrix jacchus</i>	PREDICTED: gelsolin-like isoform 1
8	259	21	10	gi 38044288	<i>Homo sapiens</i>	gelsolin isoform b
9	59	5	2	gi 194038353	<i>Sus scrofa</i>	PREDICTED: similar to alpha-1-antichymotrypsin 2
9	150	19	8	gi 27806351	<i>Bos taurus</i>	ezrin
9	53	8	3	gi 27807349	<i>Bos taurus</i>	factor XIIa inhibitor precursor
11	91	18	5	gi 15987978	<i>Oryctolagus cuniculus</i>	Chain A, S402p Mutant Of Rabbit Muscle Pyruvate Kinase
11	197	34	10	gi 194670470	<i>Bos taurus</i>	PREDICTED: pyruvate kinase, muscle
11	134	18	6	gi 31981562	<i>Mus musculus</i>	pyruvate kinase isozymes M1/M2
11	102	8	3	gi 357	<i>Bos taurus</i>	fibrinogen beta chain
11	56	10	5	gi 77736171	<i>Bos taurus</i>	hemopexin precursor
12	106	6	4	gi 167045824	<i>Callithrix jacchus</i>	ATPase, H+ transporting, lysosomal accessory protein 1 precursor (predicted)
12	72	10	2	gi 881556	<i>Mustela putorius</i>	fibrinogen, gamma A chain

12	72	7	3	gi 31092	<i>Homo sapiens</i>	unnamed protein product, PROVISIONAL elongation factor 1-alpha
13	128	22	5	gi 213021132	<i>Ovis aries</i>	pigment epithelium-derived factor
13	260	40	12	gi 52783777	<i>Equus caballus</i>	RecName: Full = Phosphoglycerate kinase 1
13	145	28	5	gi 125975938	<i>Trichosurus vulpecula</i>	CP4 protein
13	162	18	6	gi 148709584	<i>Mus musculus</i>	mCG50578 dead box helicase
13	176	18	6	gi 4758112	<i>Homo sapiens</i>	spliceosome RNA helicase BAT1
15	376	48	12	gi 145308887	<i>Ovis aries</i>	intelectin 2
15	293	52	13	gi 157886697	<i>Ovis aries</i>	intelectin-3
15	103	9	2	gi 281338720	<i>Ailuropoda melanoleuca</i>	hypothetical protein PANDA_018257 Trans-Isoprenyl Diphosphate Synthase region
15	116	26	10	gi 297481614	<i>Bos taurus</i>	PREDICTED: prostaglandin F synthase 2-like
15	148	32	11	gi 30794344	<i>Bos taurus</i>	dihydrodiol dehydrogenase 3
17	85	12	3	gi 194037097	<i>Sus scrofa</i>	carbonic anhydrase 2
17	129	33	5	gi 41406048	<i>Bos taurus</i>	chymotrypsin-like elastase family member 1 precursor
17	93	15	3	gi 291410030	<i>Oryctolagus cuniculus</i>	PREDICTED: NADPH:secondary-alcohol oxidoreductase-like
19	57	16	2	gi 77735599	<i>Bos taurus</i>	ribosyldihydronicotinamide dehydrogenase
19	152	42	5	gi 61553385	<i>Bos taurus</i>	heat shock 27 kDa protein 1
19	141	17	5	gi 3329498	<i>Mus musculus</i>	heterogenous nuclear ribonucleoprotein A2/B1
20	102	12	2	gi 114051137	<i>Bos taurus</i>	tetranectin precursor
20	111	26	4	gi 126305670	<i>Monodelphis domestica</i>	PREDICTED: similar to proliferation associated gene (pag)
20	217	52	6	gi 291415601	<i>Oryctolagus cuniculus</i>	PREDICTED: cofilin 1 (non-muscle)-like, partial
20	69	5	2	gi 296477855	<i>Bos taurus</i>	galectin-4
20	79	–	2	gi 126336522	<i>Monodelphis domestica</i>	PREDICTED: similar to RAB43
20	89	10	2	gi 296478535	<i>Bos taurus</i>	RAB35, member RAS oncogene family
20	84	20	3	gi 4389379	<i>Homo sapiens</i>	Chain A, Cdc42hs-Gdp Complex
21	107	27	4	gi 119901287	<i>Bos taurus</i>	PREDICTED: transgelin 2
21	140	14	4	gi 169163387	<i>Homo sapiens</i>	PREDICTED: similar to TRIM5/CypA fusion protein
21	183	57	7	gi 281347999	<i>Ailuropoda melanoleuca</i>	hypothetical protein PANDA_001384 Arf-1-Arf-5-like subfamily region
21	120	24	2	gi 441486	<i>Bos taurus</i>	coatomer, Clathrin adaptor complex small chain region
21	132	37	5	gi 74225050	<i>Mus musculus</i>	unnamed protein product, RhoA_like region
22	103	19	3	gi 152207470	<i>Capra hircus</i>	galectin 15
22	79	29	3	gi 4507793	<i>Homo sapiens</i>	ubiquitin-conjugating enzyme E2 N
24	54	21	2	gi 15216177	<i>Bos taurus</i>	putative 42-9-9 protein
24	72	41	4	gi 163315	<i>Bos taurus</i>	lysozyme 2a precursor
24	222	60	6	gi 34810222	<i>Capra hircus</i>	Chain A, Crystal Structure Of The Mammalian Tumor Associated Antigen Uk114
24	89	22	3	gi 73974071	<i>Canis lupus familiaris</i>	PREDICTED: similar to Ribonuclease UK114 (14.5 kDa translational inhibitor protein) isoform 3
26	55	3	2	gi 27806447	<i>Bos taurus</i>	proactivator polypeptide (prosaposin)

Table 8. (Cont.)

Gel slice ^a	MOWSE score	Sequence coverage (%) ^b	Total no. peptides ^c	GenInfo Identifier ^d	Species	Identity
Naïve abomasal environment						
7	70	4	2	gi 27806815	<i>Bos taurus</i>	plasminogen precursor
7	71	4	2	gi 67846016	<i>Ovis aries</i>	glycogen phosphorylase, liver form
7	71	3	2	gi 291395030	<i>Oryctolagus cuniculus</i>	PREDICTED: type 1 tumor necrosis factor receptor shedding aminopeptidase regulator
7	56	—	2	gi 3282771	<i>Homo sapiens</i>	actin binding protein homologue ABP-278
7	62	7	4	gi 4321127	<i>Homo sapiens</i>	IgG Fc binding protein
7	75	18	8	gi 55217	<i>Mus musculus</i>	murine valosin-containing protein
8	66	6	3	gi 1170383	<i>Cricetulus griseus</i>	RecName: Full = Heat shock protein HSP 90-alpha
8	66	—	3	gi 123681	<i>Mus musculus</i>	heat shock protein 90 beta
8	69	7	5	gi 27807263	<i>Bos taurus</i>	endoplasmic precursor hsp90 beta
8	143	9	4	gi 194223978	<i>Equus caballus</i>	PREDICTED: threonyl-tRNA synthetase
9	69	10	3	gi 31283	<i>Homo sapiens</i>	unnamed protein product
10	56	6	2	gi 157074114	<i>Bos taurus</i>	plastin-1 a
12	141	22	10	gi 118494	<i>Equus caballus</i>	RecName: Full = Retinal dehydrogenase 1; AltName: Aldehyde dehydrogenase
12	66	7	3	gi 194036227	<i>Sus scrofa</i>	Selenium-binding protein 1
12	101	10	3	gi 46485440	<i>Rattus norvegicus</i>	glucose-6-phosphate isomerase
12	85	23	6	gi 57164383	<i>Ovis aries</i>	antithrombin-III precursor
12	69	11	2	gi 1083124	<i>Ovis aries</i>	Ig mu heavy chain V region precursor – sheep (fragment)
12	134	9	3	gi 837	<i>Bos taurus</i>	unnamed protein product, RNA binding domain
13	178	19	7	gi 114051782	<i>Bos taurus</i>	4-trimethylaminobutyraldehyde dehydrogenase
13	162	15	5	gi 435487	<i>Homo sapiens</i>	aldehyde dehydrogenase (NAD +)
13	147	38	10	gi 78042564	<i>Bos taurus</i>	cytosolic non-specific dipeptidase
13	412	42	15	gi 285975	<i>Homo sapiens</i>	human rab GDI
13	176	31	12	gi 4929854	<i>Homo sapiens</i>	Chain A, Structure Of Human Placental S-Adenosylhomocysteine Hydrolase
14	224	54	4	gi 163101	<i>Bos taurus</i>	glutamine synthetase
15	233	45	11	gi 160286558	<i>Oryctolagus cuniculus</i>	Chain A, Fructose 1,6-Bisphosphate Aldolase From Rabbit Muscle
15	198	22	7	gi 229503	<i>Oryctolagus cuniculus</i>	aldolase
15	206	37	8	gi 57526769	<i>Ovis aries</i>	chymosin precursor
16	231	37	9	gi 147900301	<i>Bos taurus</i>	biliverdin reductase A
16	148	27	5	gi 149743872	<i>Equus caballus</i>	PREDICTED: similar to 3(2), 5-bisphosphate nucleotidase 1
16	133	34	6	gi 165875541	<i>Ovis aries</i>	transaldolase 1
16	137	16	5	gi 194209508	<i>Equus caballus</i>	PREDICTED: similar to MGC155110 protein, biliverdin reductase region
16	269	10	3	gi 2947100	<i>Sus scrofa</i>	15-oxoprostaglandin 13-reductase
16	341	26	8	gi 296484379	<i>Bos taurus</i>	prostaglandin reductase 1
16	150	—	3	gi 56789674	<i>Mus musculus</i>	farnesyl diphosphate synthetase
16	197	31	6	gi 77736025	<i>Bos taurus</i>	3'(2'),5'-bisphosphate nucleotidase 1

17	233	27	8	gi 115495641	<i>Bos taurus</i>	alcohol dehydrogenase [NADP+]
17	252	34	6	gi 115497074	<i>Bos taurus</i>	S-formylglutathione hydrolase
17	342	36	10	gi 300796734	<i>Bos taurus</i>	annexin A10
17	96	10	2	gi 301764565	<i>Ailuropoda melano</i>	PREDICTED: LOW QUALITY PROTEIN: isochorismatase domain-containing protein 1-like
17	108	33	4	gi 4929691	<i>Homo sapiens</i>	CGI-111 protein
17	208	22	6	gi 6754524	<i>Mus musculus</i>	L-lactate dehydrogenase A chain isoform 1
17	89	8	2	gi 74002351	<i>Canis familiaris</i>	PREDICTED: similar to caspase 6 isoform alpha preproprotein
17	378	35	9	gi 77736203	<i>Bos taurus</i>	malate dehydrogenase, cytoplasmic
17	215	19	5	gi 78042488	<i>Bos taurus</i>	adenylyl cyclase-associated protein 1
17	103	20	4	gi 126337842	<i>Monodelphis domestica</i>	PREDICTED: similar to p34-Arc
19	84	14	2	gi 4504771	<i>Homo sapiens</i>	eukaryotic translation initiation factor 6 isoform a
19	81	27	4	gi 7106387	<i>Mus musculus</i>	proteasome subunit alpha type-5
20	149	41	5	gi 240849225	<i>Ovis aries</i>	adenylate kinase isoenzyme 1
20	111	25	4	gi 2497487	<i>Sus scrofa</i>	RecName: Full = UMP-CMP kinase; AltName: Full = Cytidine monophosphate kinase
20	186	39	4	gi 28189813	<i>Bos taurus</i>	similar to translationally controlled tumor protein
20	113	20	3	gi 32401410	<i>Bos taurus</i>	immunoglobulin J chain
20	83	34	2	gi 4322114	<i>Isoodon macrourus</i>	hypoxanthine phosphoribosyltransferase HPRT-1
20	85	–	2	gi 77404180	<i>Rattus norvegicus</i>	Ras-related small GTP binding protein 4
21	111	47	6	gi 296488630	<i>Bos taurus</i>	anterior gradient 2 homolog
21	97	11	2	gi 16758368	<i>Rattus norvegicus</i>	ras-related protein Rab-14
22	118	22	2	gi 4758442	<i>Homo sapiens</i>	glia maturation factor beta
23	150	42	6	gi 114051526	<i>Bos taurus</i>	coactosin-like protein
23	103	37	3	gi 1293786	<i>Bos taurus</i>	LP2, differentiation-associated lipid binding protein
24	501	79	8	gi 253723066	<i>Bos taurus</i>	Chain A, Crystallization And Structure Determination Of Bovine Profilin
25	90	61	5	gi 5031985	<i>Homo sapiens</i>	nuclear transport factor 2
25	80	25	2	gi 1942335	<i>Bos taurus</i>	Chain C, Ternary Complex Of A Calcineurin A Fragment, Calcineurin B, Fkbp12 And The Immunosuppressant Drug Fk506 (Tacrolimus)
25	60	25	2	gi 197724894	<i>Homo sapiens</i>	Chain A, Crystal Structure Of E60q Mutant Of Fkbp12 – FKBP-type
26	69	26	3	gi 545707	<i>Bos taurus</i>	peptidyl-prolyl cis-trans isomerase Cap1 = S-100-related calcium-binding protein

The most abundant transcript in each larval dataset was a *B. malayi* hypothetical protein homologue with similarity to a checkpoint-like protein from *Helicoverpa armigera* (cotton bollworm) which shows increased expression during larval molting (Dong *et al.* 2007). Here, the larvae in both treatments were exsheathing during the incubation period and the presence of high levels of this transcript may reflect this process.

Transcriptomic analysis of the parasitic nematode *Strongyloides ratti* (O'Meara *et al.* 2010; Thompson *et al.* 2008) showed an increase in expression of an IDA family member (*ida-1*) and a small heat shock protein family member (*C. elegans* HSP 17) in response to host immunity and high immune pressure respectively, results comparable to those presented here. Interestingly, in *S. ratti unc-54* and a C-type lectin family member (*clec-200*) were also upregulated in response to host immunity, whereas these transcripts were more abundant in *T. circumcincta* larvae exposed to a naïve host environment. The *S. ratti* transcriptomic study was carried out 12 days after infection as opposed to after 4 h exposure to a naïve or immune environment, which may explain the differences observed.

KEGG biological pathways with a statistically significantly increased mean proportion of Isotigs in the immune-exposed larval sample included carbohydrate and energy metabolism, and biosynthesis of alkaloids and secondary metabolites. An increase in energy metabolism could represent an increased requirement for energy to mount responses to the host environment. The biosynthesis of alkaloids, phenylpropanoids and other secondary metabolites is known to be involved in response to environmental stress in plants (Iriti and Faoro, 2009), and herein could be representative of a stress response in the immune environment-exposed larvae accompanied by contemporaneous adjustment in metabolic pathways.

The method employed here to create the abomasal environments *in vitro* will not result in an exact replica of the physiological situation that the larvae encounter *in vivo*. Analysis of the protein profiles of these abomasal samples showed differences between the environments created using the abomasa of naïve and immune sheep, these being comparable to the results obtained from in-depth quantitative analysis of the equivalent abomasal tissue (Athanasiadou *et al.* 2008; Alan Pemberton, *personal communication*). One of the proteins with the highest number of non-redundant peptides identified from immune sheep was clathrin, a prominent protein component of cellular protein transport vesicles with the formation of a clathrin coat being fundamental to the mechanism of vesicle budding as well as receptor sorting and transport specificity (McDowall, 2007). Proteins which are known to be upregulated in the abomasa of immune sheep and which are thought to play a role

in vivo in local immunity, such as intelectin-2, galectin-15, IgE and gelsolin-b, were all detected exclusively in the immune abomasal environment sample. Sheep intelectin-2 (*Itln-2*) expression is upregulated in the abomasa of immune sheep following challenge compared to naïve sheep undergoing a primary infection and immunolocalizes to abomasal mucus neck cells and gastric mucus surrounding developing larvae (French *et al.* 2008). *Itln-2* co-purifies with the mucin *Muc5a* from gastric mucosa to alter the rheological properties of the mucus, which could potentially reduce larval penetration to the underlying mucus layer (Pemberton *et al.* 2011). Galectin-15 (*ovgal11*) shows greatly increased expression in the abomasal tissue of sheep infected with *H. contortus* compared to uninfected tissue, and the mRNA appears more rapidly in previously sensitized sheep compared to naïve sheep (Dunphy *et al.* 2000). Galectin-15 protein is also secreted into the lumen and detected in mucus where it may interact with carbohydrate motifs such as mucins. Increased levels of locally produced IgE have been observed in previously infected sheep undergoing challenge infection when compared to naïve sheep undergoing a primary infection (Huntley *et al.* 1998), and gelsolin levels in ovine gastric lymph have been demonstrated to decrease *in vivo* in naïve sheep undergoing a primary infection but not during challenge of immune sheep (Goldfinch *et al.* 2008). Gelsolin can function as an actin scavenger, possibly binding free actin released from damaged cells to prevent actin-polymerization and subsequent detrimental effects on mucus viscosity (Sun *et al.* 1999; Goldfinch *et al.* 2008). It is also known to be down-regulated as cells become less differentiated, for example during replacement of acid-producing parietal cells with non-differentiated non-acid secreting cells as is seen in the abomasum during *T. circumcincta* infection, and may also have a role in the regulation of mucus secretion from goblet cells (Vandekerckhove *et al.* 1990; Ehre *et al.* 2005). Detection of these proteins exclusively in the immune abomasal environment sample suggests that the abomasal environment fluids used for larval culture here were at least partly representative of the soluble abomasal proteome.

An astacin-like metalloprotease was identified by Smith *et al.* (2009) in the excretory/secretory (ES) products of *T. circumcincta* larvae recovered 1 day post-infection of naïve sheep; however, transcript for this protein was not detected in either of the larval datasets generated here. It is possible that production of this protein had not yet commenced within the 4 h of exposure to the abomasal environment as compared to the 24 + h exposure described by Smith *et al.* (2009).

In conclusion, this paper defines the major transcriptional changes in *T. circumcincta* upon encountering either a naïve or an immune ovine

host environment. Statistically significant differences in mean transcript levels between the 2 larval populations were detected for several genes with possible roles in sensory capabilities and response to the differing environments. The larvae exposed to the immune environment are encountering conditions known to lead to larval inhibition, therefore the possibility exists that the transcriptional changes observed may relate to larvae entering a state of arrest. Further work could focus on localization and functional analysis of these proteins to further determine the response of the parasites to varying host environments and concomitant effects on parasitic behaviour and development.

DATA

454 sequencing data are available in the Sequencing Read Archive (SRA), Accession number ERP000810, <http://www.ebi.ac.uk/ena/data/view/ERP000810>.

Assembled transcript data are available via the PartiGene database http://genepool.bio.ed.ac.uk/GP_Partigene/2010038_AileenHalliday_AH15AH16/ (Parkinson *et al.* 2004).

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8 Discussion

8.1 The need to maintain productivity

With the ever-growing world population estimated to reach 9.1 billion by 2050 (United Nations News Centre 2005), there is an ever-growing demand for food. Overall food production will need to rise by 70 % to meet this demand, and in developing countries will need to almost double. Livestock production is currently one of the fastest growing sub-sectors in agriculture and the demand for meat is projected to rise faster than the demand for cereals as the world population becomes more urbanized and wealthy (Food and Agriculture Organisation 2009). Food security, the ability of everyone to have access to safe, nutritious food at all times (World Health Organisation 2013), is an important area to address within this context. Within the EU, environmental changes are exacerbating the already substantial economic damage helminth parasites inflict upon the agricultural industry, prompting the European Commission to fund extensive international collaborative research projects aimed at investigating sustainable strategies to counteract the effect of helminth infections in ruminants (European Commission 2012). Nematode parasites are also deemed to be the greatest threat to animal health and productivity in developing regions (Waller 2006), and as eradication does not seem a viable option focus must be changed to concentrate on methods of parasite management. Traditional anthelmintics will continue to play an essential role in

nematode control in the immediate future, but the search is on for sustainable alternative methods of control to complement the existing drugs. One such proposed control method is vaccination.

Much of our understanding of how the host immune system regulates infection with gastrointestinal nematodes has been derived using murine infection models. These studies have established that immunity to intestinal-dwelling worms is critically dependent on a type 2 cytokine response controlled by CD4⁺ T-helper type 2 cells that secrete the cytokines IL-4, IL-5, IL-9 and IL-13 (Artis 2006). Artis (2006) noted that the immune effector mechanisms elicited by type 2 cytokines in the gut microenvironment that lead to worm expulsion remain elusive. Host intestinal epithelial cells were identified as having central importance with novel type 2 cytokine-dependent pathways and effectors being implicated in the mechanisms of expulsion. Relatively novel molecules identified for further attention included intelectins and resistin-like molecules, as well as how cytokines regulate intestinal epithelial cell turnover. Identifying how the mammalian immune response fights gastrointestinal nematode infections is providing new insights into host protective immunity. Harnessing these discoveries, coupled with identifying what the targets of these responses are within parasitic nematodes, offers promise in the design of a new generation of anti-parasitic drugs and vaccines.

8.2 Feasability of vaccine approach

The success of native gut glycoproteins in immunising sheep against the gastrointestinal nematode *Haemonchus contortus* (LeJambre *et al* 2008; Smith *et al* 1994) demonstrated that sub-unit vaccination against nematodes was a feasible approach for control of these parasites in ruminants. The success of cross protection studies carried out in calves using vaccination with *Haemonchus contortus* gut antigens followed by challenge infection with either *Haemonchus contortus* or *Haemonchus placei* also demonstrated the potential use of the *H. contortus* gut antigen approach in multiple species (Bassetto *et al* 2011). A novel simplified *H. contortus* gut membrane protein batch preparation method utilised in extensive field trials in Australia, South Africa and Brazil, has also demonstrated the possibility of this approach becoming economically viable and fitting with on farm practices, and commercial vaccine registration is in progress (Smith WD, personal communication). However limited work conducted up to the time of this thesis was not encouraging when considering the same approach in non-obligate blood feeding nematodes despite adults of *Ostertagia ostertagi* and *Teladorsagia circumcincta* having homologues of the host-protective *H. contortus* intestinal proteins (Smith *et al* 2001). However, host immunoglobulin could be detected in homogenates of adult worms or fourth stage larvae of *O. ostertagia* and *T. circumcincta* and was present in the intestine indicating that immunoglobulin had been ingested, even by the non-blood-feeding species (Murray and Smith 1994). This finding suggested that the

Ostertagia and *Teladorsagia* may be susceptible to vaccination by the gut antigen approach.

Smith *et al* (Smith *et al* 2000) used lectin affinity chromatography to enrich glycoproteins from detergent extracts of adult *Ostertagia ostertagi* membranes. SDS-PAGE indicated that the protein profile of these fractions closely resembled the equivalent *Haemonchus contortus* proteins derived from the intestinal cell microvillar membranes and which are highly protective when used as antigens (Knox and Smith 2001). In calves vaccinated with pooled peanut and ConA binding detergent soluble fractions of *Ostertagia* and given a single dose of infective L3, moderate (30-50%) reductions in egg output were observed in vaccinates compared to controls although the worm numbers were unaffected. Sheep immunized with the same *Ostertagia* fractions were highly protected (>80% and 57% reductions in egg output and worm burdens respectively) in two trials against *Haemonchus* challenge. Combined, these data indicated that the adult-derived gut antigen approach to vaccination would not be sufficiently effective to control *Ostertagiosis* in calves. Around the same time, the *O. ostertagi* equivalent of the cysteine protease-enriched fraction from adult *H. contortus* (designated Thiol Sepharose binding proteins (TSBP; (Knox *et al* 2005)) was evaluated against homologous challenge in calves and was ineffective although the equivalent fraction from adult ES did induce protective immunity in calves given a trickle challenge infection with L3s (Geldhof *et al* 2002).

While detergent soluble glycoprotein antigen preparations from adult *O. ostertagi* did stimulate a degree of protective immunity in calves against homologous challenge (Smith *et al* 2000), this would not be sufficient to control infection in the field based on epidemiological predictions and computer modelling (Barnes *et al* 1995). Although work by Murray and Smith (Murray and Smith 1994) did indicate that the adult worm ingested host immunoglobulin, this was in notably less amounts than that observed for adult *Haemonchus contortus*. Within the abomasal glands, the larval parasite grows very rapidly (~10-fold) in the transition from fourth stage larva (L4) to the immature adult. In addition, its position raises the possibility that it would be likely to ingest more host serum proteins, including immunoglobulin, than the adult stage. Hence, the hypothesis was that targeting intestinal proteins in the L4s may provide a source of more efficacious vaccine antigens than the equivalent proteins from the adult.

This hypothesis was tested by isolating L4 *O. ostertagi* antigens in a manner similar to that used for the *Haemonchus* gut antigens, and is described in Paper 4 herein (Halliday and Smith 2010). Vaccine trials against homologous challenge using the L4 detergent soluble ConA binding extract resulted in average reductions of up to 85% and 64% in total egg output and worm burdens respectively. Immunolocalisation studies are required to confirm that some or all of these antigens are derived from the larval intestine, however the extract used would appear to contain protective antigens. In addition, serology would be required to define whether or not these antigens are recognised by

the natural immune response to infection, thus defining if they are true hidden antigens of the same nature as the protective *Haemonchus contortus* gut antigens.

While the above studies indicate that vaccination would be a feasible objective for the control of *O. ostertagi* in cattle, vaccine trial data for *T. circumcincta* have been less promising. Several attempts have been made to protect lambs against *T. circumcincta* infection with TSBP from the adult and L4 stage parasite with only moderate, ~30% reductions in worm burdens and egg outputs (Knox D.P. unpublished results). In Paper 5 herein (Halliday and Smith 2011) lambs were immunised with a ConA-binding antigen fraction prepared from *T. circumcincta* L4s, a fraction proposed to contain similar antigens to that which conferred protective immunity to calves against *O. ostertagia* challenge (Halliday and Smith 2010), and likely to contain a broader range of possible protective antigens than the TSBP fraction outlined above. The vaccine induced high-titre serum antibody responses in two trials but no significant reduction in either egg count or worm burdens was observed in the vaccinated groups compared to the controls in either trial. This result indicated that detergent-soluble, ConA-binding extracts prepared from *T. circumcincta* fourth-stage larvae did not contain significantly protective antigens.

The lack of efficacy of gut-derived membrane bound intestinal antigens from both L4 and adult *T. circumcincta* as vaccines focused attention on natural immunity in lambs - the immune effector mechanisms as well as the antigens stimulating them.

8.3 Natural immunity

The relative lack of exposure of non blood-feeding parasites to host immunoglobulin may be a contributing factor in why gut antigen immunisation, which stimulates high levels of circulating serum IgG, may be unsuccessful. An understanding of how to stimulate a mucosal response which more closely relates to the natural immune response mounted to these parasites, such as local production of IgA, may well be essential to the success of vaccinating against parasites residing in mucosal tissue (Sedgmen *et al* 2006; Sedgmen *et al* 2004). This will most likely include investigation of delivery methods such as intra-nasal and intra-rectal to target delivery of antigen to appropriate immune effector tissue, and also investigation into appropriate adjuvants. Some success has already been achieved against the sheep intestinal parasite *Trichostrongylus colubriformis*, with native or recombinant antigen delivered across the rectal epithelium inducing immunity at varying levels against homologous challenge (McClure 2008) adding hope to the prospect of mucosal immunisation. While intra-rectal immunisation is invasive and can be technically difficult and time consuming, requiring specialist equipment to reach the correct tissue, and as such may not be a realistic approach for on-farm practice, the success of intra-rectal immunisation does demonstrate the potential for effect mucosal immunisation with appropriate antigen targeting. In general terms, animals with the highest serum IgA responses also showed the highest degree of protection. However, the author did caution that the observed correlations and

antibody titres themselves would be heavily influenced by the time of sampling and should be interpreted with caution.

It is generally accepted that an understanding of natural immunity is essential to inform vaccine development and gastrointestinal parasite control (Miller 2008). Papers 1, 2 and 3 contained herein, published in 2007, 2009 and 2010 respectively, added to the knowledge at the time regarding the composition and kinetics of the local immune response.

In papers 1 and 2 (Halliday *et al* 2007; Halliday *et al* 2009) groups of yearling sheep were trickle infected with *T. circumcincta* for 8 weeks and then drenched. They, and worm free controls, were then challenged with 50 000 *T. circumcincta* larvae. In paper 1 (Halliday *et al* 2007), fewer parasites and a greater proportion of early fourth stage larvae were recovered from previously infected sheep compared to controls. Worm loss and arrested development were evident by 5 days after challenge whereas growth retardation of developing worms was observed by day 10. In the previously infected sheep a secondary IgA response was observed in the efferent gastric lymph from 5 days post-infection. The concentration of IgA in lymph increased five-fold 8 days post-challenge and the timing of the response suggested that it occurred too late to have been the cause of worm loss or arrested development, though it may have retarded the growth of developing parasites.

In paper 2 (Halliday *et al* 2009), a similar experimental design was used but the intention was to focus on the kinetics and phenotype of the local cell traffic in response to *T. circumcincta* infection via serial sampling of efferent gastric

lymph. Again, reduced worm burdens were evident in the previously infected animals as early as 2 days after challenge, somewhat earlier than the 5 day interval noted above (Halliday *et al* 2007). A blast cell response, consisting of both T and B lymphocytes, was observed in both groups of sheep in the gastric lymph but occurred more rapidly in the previously infected, immune animals. CD4+, CD8+ and CD25+ blast cell output peaked at day 3 in the previously infected animals, whereas CD21+ blast cell output peaked slightly later at day 5. In the control group the peak output of all phenotypes of blast cells occurred more slowly, peaking 10 days after infection. The early blast cell response, which preceded the elevation in lymph IgA noted in paper 1 (Halliday *et al* 2007) may develop sufficiently early to indicate that this response impairs parasite establishment and maintenance within the host.

In the third study of this series (Halliday *et al* 2010), 5 month-old lambs were exposed to the same infection regime with the aim of unraveling the basis for the long held belief that young (<5month old) lambs were more susceptible to challenge infection than older (~10 month old or adult) counterparts (Colditz *et al* 1996; Smith *et al* 1985). No significant differences were noted in the response to challenge in the two age groups. From 10 days post infection, fewer parasites were recovered from the previously infected lambs, and secondary cellular and humoral responses were observed in the gastric lymph. CD4+ and CD25+ T lymphoblast traffic peaked on day 3, followed by CD21+ and IgA+ lymphoblasts on day 5, representing T cell activation, and B cell activation and antibody production respectively. There was also an increase in total and parasite

specific IgA concentrations which peaked on day 6 in the previously infected lambs. Similar peaks in lymphoblast output were not observed until days 10-12 in the control lambs. These data were highly comparable with those from yearling sheep subjected to an identical infection-challenge regime (Halliday *et al* 2009) but, notably, contrasted with data from similar experiments in the 1980s when 4.5 -month-old previously infected lambs were more susceptible to and had much weaker immune responses to challenge than 10-month-old sheep (Smith *et al* 1985). In the earlier studies previously infected 10 month sheep contained relatively fewer challenge worms, and a greater proportion of these were arrested than in 4.5 -month-old lambs which had received an identical immunising regime. That an age difference was not evident in the experiments described herein could be due to several factors including sheep breed and level of nutrition, however both possibilities were deemed as being unlikely (Halliday *et al* 2010). It was concluded that the contrasting results may reflect the different infection regimes used in the two series of trials. In the 1980s, 2000 *T. circumcincta* L3 were given to the previously infected sheep 5 days a week whereas in the recent series of trials this dose was administered only three times per week, i.e. the recent sheep received only 60% of the dose given in the 1980s. Importantly, there was no evidence from the recent trials with the lighter trickle infection to support the idea that one or more components of the immune response were defective in lambs. It is quite possible that only older, more resilient sheep are able to respond adequately to heavier infection regimes.

While the acquisition and continued boosting of natural immunity through low-level parasite exposure is essential for maintaining immunity, an understanding of natural immunity may not be a prerequisite for development of a successful vaccination strategy. As noted earlier, hidden antigens which are not seen by the host immune system during the course of natural infection can be strong vaccine targets and provide protection to the host while the development of natural immunity, driven by exposure to natural (also known as conventional) antigens occurs (Munn 1997). The protective *H. contortus* gut antigens H11 and H-gal-GP are both examples of hidden antigens (Smith *et al* 1993; Smith *et al* 1994).

Furthering our understanding of the biology of both the host and the parasite, and their interactions, undoubtedly informs the development of control strategies, drugs and vaccines. However in the case of successful vaccination we do not always fully understand the nature or interaction of essential host and parasite factors conferring immunity.

8.4 Transcriptomic studies

Paper 6 contained herein (Halliday *et al* 2012) describes a transcriptomic study of gene expression in third larval stage *T. circumcincta* using next-generation 454 sequencing. Comparative analysis of the sequencing data was used to determine genes that were differentially regulated in larvae exposed to an immune host environment compared to those exposed to a naïve host

environment. Careful consideration had to be given to the lack of biological repeats in this study, resulting in development of a conservative statistical model to detect differentially expressed transcripts. The addition of the complementary liberal model of analysis revealed further potentially differentially expressed candidates of biological relevance that may be worth further investigation.

Eighteen transcripts were identified under the conservative model as being differentially expressed between immune- and naïve- exposed larvae. O'Meara et al (O'Meara *et al* 2010) also found that changes in expression occurred in only a small proportion of genes following exposure of *Strongyloides ratti* to hosts of differing immune status. Eleven of the 18 clusters identified as being differentially expressed in the current study showed greater read count abundance in larvae exposed to the immune environment. These included genes involved in neuropeptide processing and potential immunosuppression (PGAL-1 protein homologue), neuropeptide transport (IDA-1 homologue) and protection from oxidative stress (HSP16-2). It would appear that even after only 4 hours of larval exposure to the immune environment, and using a conservative statistical model, statistically significant changes in the expression of biologically relevant genes can be detected. These genes would appear to be involved in the response to increased immune pressure from the host. Future work using this system will focus on further quantitative analysis of the lead candidates as well as immunolocalisation studies.

Transcriptomic studies using next generation sequencing technologies, as described in paper 6 contained herein, have a number of merits and drawbacks.

Large amounts of data can be generated, with massive potential for comparative analyses within well designed experiments. Placing the data in publicly accessible databases also adds to the availability of data for everyone, and will potentially assist in genomic and post-genomic annotation. The main drawback to carrying out transcriptomic studies using next-generation sequencing at the time of writing this paper was cost. At several thousand pounds for each dataset generated, the cost of carrying out biological replicates and extensive experiments could be prohibitive for most. Dicker *et al* (Dicker *et al* 2011) and Cantacessi *et al* (Cantacessi *et al* 2010b) have published comparative transcriptomic studies using methods similar to those used in this thesis, also without biological repeats. Potentially the cost of this technology will decrease over time, as is often observed when new technologies are developed further and become more widely available. The large datasets generated, potential gold mines of information, can also be very labour intensive to analyse and this would also increase given multiple datasets.

8.5 Proteomic studies

Proteomic analysis of the abomasal environment samples described in Paper 6 (Halliday *et al* 2011) was carried out via liquid chromatography-electrospray ionization- tandem mass spectrometry (LC-ESI-MS/MS), to ascertain that the environment for *in vitro* larval exposure was partially representative of that encountered *in vivo*. The peak list file generated from the resultant data was

submitted to the NCBIInr database using the MASCOT search engine. Strict parameters were adhered to when analysing the resultant protein matches: they were required to have a MOWSE score of over 45, at least 2 non-redundant peptides were required to hit the protein, and there had to be at least 4 consecutive *b* or *y* ions within each peptide. This approach yields high quality data presented in line with the guidelines published by Molecular and Cellular Proteomics (Molecular and Cellular Proteomics 2007). Previous LC-ESI-MS/MS proteomic analysis of abomasal mucus washings from sheep immune to *T. circumcincta* identified greater levels of galectin 15, intelectin and gelsolin b than in mucosal washings from naïve sheep (Athanasiadou *et al* 2008). Galectin-15 and intelectin have been proposed to interact with mucins in the lumen of the gut thereby effecting the rheological properties of mucus and reducing larval penetration (Dunphy *et al* 2000; French *et al* 2008; Pemberton *et al* 2011). Gelsolin can act as an actin scavenger reducing the detrimental effects on mucus viscosity of polymerisation of free actin released from damaged cells, and may also be involved in regulation of mucus secretion from goblet cells (Ehre *et al* 2005; Sun *et al* 1999). Goldfinch *et al* (Goldfinch *et al* 2008) also identified gelsolin as being more abundant in the efferent gastric lymph of immune sheep after infection compared to naïve sheep, and it was reassuring to see these proteins represented solely in the immune abomasal environment samples that the larvae were exposed to during the transcriptomic study described herein (Halliday *et al* 2012). Clathrin, which is involved in vesicle formation for molecular transport, was also identified in the immune abomasal environment and may represent neurotransmission and signal

transduction following parasite infection (McMahon and Boucrot 2011).

Qualitative analysis by means of *in-silico* subtraction and manual curation revealed that approximately 10% of the proteins in each environment were unique to that sample. Quantitative proteomic analysis of the environments could potentially provide an even greater insight into the differing secretome of immune and naïve abomasal tissue.

8.6 Vaccine production

Despite the success of vaccinating cattle against *O. ostertagi* described herein, the time consuming and expensive method of collecting L4 starting material is a major constraint to vaccine development. *In vitro* larval culture beyond the L3 stage is complex and inconsistent (Douvres and Malakatis 1977), therefore fourth stage larvae must be harvested from calves. At least 20 previously worm-free calves were infected then slaughtered to produce enough fourth stage larvae for each round of antigen production.

The most attractive method of producing a commercially viable vaccine against nematodes would be to produce large amounts of recombinant protein (Smith 2008). The success of vaccination against the parasites *Boophilus microplus* in cattle (Rand *et al* 1989), *Taenia ovis* in sheep (Harrison *et al* 1996) and *Trichostrongylus colubriformis* in sheep (McClure 2008) using recombinant proteins has demonstrated the feasibility of this approach as a control strategy for complex metazoan parasites. However, to date, there has been little success

vaccinating against abomasal nematodes using recombinant proteins. As detailed in Table 1, recombinant *Ostertagia* proteins have so far failed to protect cattle against homologous challenge (De Maere *et al* 2005; Geldhof and Knox 2008; Vercauteren *et al* 2004; Vercauteren *et al* 2006). Recombinant versions of components of the protective *H. contortus* H-gal-GP complex also failed to induce protective immunity in sheep (Cachat *et al* 2010). This lack of success with recombinant protein compared to native protein may be due to incorrect folding, lack of enzyme activity or incorrect post-translational modifications such as glycosylation, all of which may be species-specific and carried out incorrectly in alternative expression vectors. *Caenorhabditis elegans* is under investigation as a potential expression vector for nematode proteins, as it is likely to produce recombinant proteins with a structure and glycosylation pattern similar to that in parasitic nematodes (Murray *et al* 2007) which may aid in the development of protective antibody production following immunisation.

Recent success has been achieved using recombinant protein produced in *E. coli* to vaccinate mice against the thread worm *Strongyloides stercoralis* (Abraham *et al* 2011) however these parasites migrate to the intestine via the circulatory system, which may contribute towards the success of vaccination through increased exposure to host immunoglobulin.

An additional limiting factor in producing recombinant vaccines is that identification of the protective antigens is required, and the L4 extract used in the *Ostertagia* trials described herein is a complex mix of proteins. Preliminary

identification of the components is underway, however more complete genome annotation is required before full classification can be completed.

8.7 Phage display library biopanning

A new approach to screening for protective antigens is via peptide phage-display library biopanning (Ellis *et al* 2011; Wei *et al* 2011). If recombinant proteins are being produced with the wrong structural confirmation and this is limiting their efficacy, the screening of peptide epitopes for those which potentially mimic the structure of protective antigenic epitopes may provide an alternative source of protective antigens. Significant reduction in *Fasciola hepatica* worm burden and egg output has been achieved following vaccination of sheep with a Cathepsin L epitope mimic (Villa-Mancera *et al* 2008), and Wei *et al* (2011) achieved substantial protection against *Trichinella spiralis* in mice using a synthetic peptide identified using this method.

8.8 DNA vaccination

DNA vaccination in which plasmid DNA containing foreign antigen DNA is injected into the host where it is transcribed, expressed, and an immune response mounted to the foreign protein, is another technology under investigation for vaccination of livestock against parasitic nematodes (Alarcon *et al* 1999; Smooker *et al* 2004). Promising results have been obtained using DNA vaccination against *Haemonchus contortus* in goats. An *H. contortus*

glutathione peroxidase DNA vaccine stimulated an immune response in goats and reduced worm burdens and egg counts by 36% each (Sun *et al* 2011). DNA vaccine encoding the *Haemonchus* H11 antigen reduced egg counts and worm burdens by 45% and 38% respectively, and this protection was increased to a 57% and 47% reduction respectively when the H11 DNA vaccine was given in conjunction with interleukin-2 DNA vaccine (Zhao *et al* 2011), demonstrating the potential flexibility of this type of vaccination to incorporate factors which could skew the immune response in the desired direction, eg towards a Th2 type response. Partial protection was also observed in goats following *H. contortus* cysteine protease DNA vaccination (Muleke *et al* 2007).

9 Summary

The increasing prevalence of multiple anthelmintic-class resistance in gastrointestinal nematodes highlights the need to continue the search for the best possible control strategy for these parasites. At least in the short term, reliance on chemical anthelmintic drugs will continue and best practices need to be determined and adopted to optimise the longevity of these drugs. In the long term vaccination is an attractive alternative, but extensive research and development is still needed to reach this goal. Protective antigens remain to be elucidated fully and much development will be necessary to make a successful vaccine commercially viable and within the reach of livestock producers who would benefit from it. The ultimate goal of vaccination would be to create an affordable multi-valent vaccine which would confer protection against several species of parasite.

Transcriptomic studies aid our understanding of the actions of the parasite within the host, and the development of new technologies continues to advance this.

The papers contained within this thesis have contributed to our knowledge of natural ovine immunity to *Teladorsagia circumcincta*, demonstrated the potential of *Ostertagia ostertagi* L4 antigens as a vaccine strategy in cattle and highlighted differences with L4 antigen vaccination of sheep against *T. circumcincta*, and made steps on the road towards elucidating host-parasite

interactions during *T. circumcincta* infection of ovine hosts of differing immune status.

The continued sharing of knowledge on host immunity, parasite biology and technical advances will hopefully lead to the future development of a sustainable control strategy for parasitic nematodes, combining all approaches necessary to maximise production and animal welfare.

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